THE ROLE OF MATER IN CYTOPLASMIC LATTICE FORMATION, ENDOPLASMIC RETICULUM DISTRIBUTION, AND CALCIUM HOMEOSTASIS IN MOUSE OOCYTES

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Ca^{2+} oscillation is hallmark of mammalian fertilization that is thought to mediate egg activation process, and dramatic targeting of the endoplasmic reticulum (ER) to the microvillar subcortex, is known to provide calcium pools for subsequent activation and embryo development. While the mechanisms by which Ca^{2+} oscillation govern later embryonic development are poorly understood, emerging evidences indicate the critical role of Ca^{2+} oscillation in embryonic development, for example, by regulating recruitment of maternal mRNAs.

Mater (Nlrp5) and *Padi6* are oocyte-and early embryo-restricted maternal effect genes that are required for embryonic development beyond the 2-cell stage. Interestingly, PADI6 localizes to, and is required for the formation of oocyte-specific structure termed cytoplasmic lattices (CPLs). Based on similarity between MATER and PADI6, we hypothesized that MATER is also required for CPL formation. Indeed, ultrastructural analysis of $Mater^{tm/tm}$ oocytes show ~90% reduced volume of CPLs and immuno-EM demonstrates the localization of MATER to CPLs, indicating MATER as a component of CPLs. Interestingly, Tubulin was also identified as a CPL component and that Tubulin interacts with PADI6. Given that Tubulin drives organelle movement, the role of Tubulin-PADI6-CPLs in organelle distribution during oocyte maturation was demonstrated.

Given above observations, we further hypothesized that *Mater* plays a role in ER distribution and function, Ca^{2+} homeostasis in oocytes. We first investigated ER distribution by microinjecting DiI into oocytes and found that, as opposed to WT oocytes, the ER in metaphase-II *Mater*$^{tm/tm}$ oocytes failed to concentrate around the spindle and cortical clusters were reduced. As cortical clusters are thought to be important for Ca^{2+} oscillation, then we tested if the pattern of Ca^{2+} oscillation is altered in *Mater*$^{tm/tm}$ oocytes after *in vitro* fertilization. Intriguingly, *Mater*$^{tm/tm}$ oocytes show altered Ca^{2+} oscillation pattern, lower amplitude and higher frequency. Then we demonstrated the reduced amount of intracellular Ca^{2+} stores in *Mater*$^{tm/tm}$ oocytes, in part, contribute to such phenotype. Finally, the localization of IP_3R-I, an essential player in releasing Ca^{2+} from ER, was altered in supporting of ER distribution. Taken together, this observation support the hypothesis that MATER is required for CPL formation and is involved in ER distribution and Ca^{2+} homeostasis in oocytes.

BIOGRAPHICAL SKETCH

Boram Kim was born in Seoul, Republic of Korea in 1981. She grew up in a loving family with parents, her older sister, younger brother, grandmother, and relatives. She graduated from Yonsei University, Republic of Korea with bachelor's degree in Biotechnology and bachelor's degree in Business administration majors in 2005. She had a chance to study in University of California, Santa Barbara from Fall 2003 to Summer 2004 as an exchange student. She became interested in Biomedical sciences through the coursework and research experience there going to Graduate school, which took her to the field of Comparative Biomedical Sciences at Cornell University in Fall 2006.

I dedicate my dissertation to my parents.

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LIST OF ABBREVIATIONS

ADAMs	A disintegrin and metalloprotease domain
ARTs	Assisted Reproductive Technologies
Astl	Astacin-like metalloendopeptidase (Ovastacin)
AURKA	Aurora kinase A
bHLH	basic helix-loop-helix
CIM	Confocal immunofluorescence microscopy
COC	cumulus oocyte complex
CPEB	Cytoplasmic polyadenylation element
CPLs	Cytoplasmic lattices (lattices)
DAG	Diacylglycerol
DiI	dicarbocyanine dye DiI_{18}
DNMT1	DNA methyltransferase 1
DPPA3	Developmental pluripotency-associated protein 3
EGA	Embryonic genome activation
ER	Endoplasmic reticulum
FIGLA	Factor in the germline alpha
FILIA	daughter in Latin
FLOPED	Factor located in oocytes permitting embryonic development
FPLC	Fast protein liquid chromatography
GPI-APs	Glycosyl-phosphatidylinositol-anchored proteins
GV	Germinal vesicle
GVBD	germinal vesicle breakdown

hCG	Human chorionic gonadotrophin
HRP	Horseradish peroxidase
HSPA5	Heat shock 70 kDa protein 5
IEM	Immuno-electron microscopy
IL-1b	Interleukin-1B
IP_3	1,4,5-trisphosphate
IVF	In vitro fertilization
LD	Lipid droplet
LRR	Leucine rich repeat
MAD2	Mitotic arrest deficient 2
MATER	Maternal antigen that embryos require (mother in Latin)
MEG	Maternal effect gene
MTs	Microtubules
MTOCs	Microtubule organization centers
MII	metaphase-II
NAP1	Nucleosome assembly protein 1
NLRP	NACHT, LRR and PYD domains-containing protein
NPM2	Nucleoplasmin 2
OET	Oocyte to embryo transition
PADI6	Peptidylarginine deiminase
PFA	Paraformaldehyde
PGC	Primordial germ cell
PHD	Plant homeobox domain

Pig-a	Phosphatidylinositol glycan class-A
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PKM	Protein kinase M
PLC	PhospholipaseC zeta
PLK1	Polo-like kinase 1
PMSG	Pregnant mare serum gonadotrophin
PYD	Pyrin domain
RanBP5	Ran-binding protein 5
SAGE	serial analysis of gene expression
SAS1B	Sperm acrosomal SLLP1 binding
SCMC	Subcortical maternal complex
SEM	Standard error of the mean
SERCA	Sarco.endoplasmic reticulum Ca^{2+}-ATPase
SLLP1	Sperm lysozyme like protein 1
TEM	Transmission electron microscopy
TLE6	Transducin-like enhancer of split 6
Triton	Triton X-100
Zar1	Zygote arrest 1
ZP	Zona pelllucida

CHAPTER I

INTRODUCTION

1. Overview of mammalian oogenesis and preimplantation embryogenesis

Primordial germ cells (PGCs), diploid precursors of eggs, give rise to oogonia that mark the start of mitosis upon arrival in the female genital ridge. The oogonia mitotically divide and become primary oocytes upon entering meiosis. The primary oocytes remain arrested in prophase I of meiosis, defined as the germinal vesicle (GV) phase. Perinatally, primary oocytes are surrounded by a single layer of flattened granulosa cells to form primordial follicles. The granulosa cells change into cuboidal in primary follicles and then they proliferate to form multiple layers in secondary follicles. As oocytes continue to grow, they produce and accumulate substantial amounts of mRNA and proteins. Hormones stimulate the formation of tertiary antrum follicles with antrum and cumulus oophorus. Under the influence of hormones, oocytes resume meiosis I, go through germinal vesicle breakdown, and form secondary oocytes. Oocyte maturation proceeds to meiosis II and re-arrests at the metaphase II (MII) stage until fertilization. Upon ovulation, fertilization occurs in the oviduct and the mature egg is activated by sperm, thereby initiating the resumption of meiosis and the completion of cytoplasmic maturation (1). After fertilization, the egg is reprogrammed into a totipotent zygote. The union of paternal and maternal haploid genome forms a diploid genome at the pronuclear (1-cell) stage and mitotic cell division begins. Following the first three cleavage divisions, the 8-cell embryo undergoes compaction, generating a tightly organized cell mass called the morula. Differentiation of the morula results in the blastocyst which is composed of a pluripotent inner cell mass and the trophectoderm. The late blastocyst will then implant in the uterus.

2. The role of maternal effect genes in egg-embryo transition

The quiescence of transcription between meiotic maturation in oocytes and embryonic genome activation (EGA) warrants an essential role of accumulated maternal transcripts and proteins that are critical for successful preimplantation development (2) (Fig. 1). The role of maternal effect genes in embryo development and axis formation is well known in lower species (3). However, mammalian maternal effect genes have been identified only recently and are a developing area of research. EGA occurs when embryonic genes start to be transcribed, though the timing of the EGA varies among species. It takes place at the morula stage in sheep and rabbits, the 4-8 cell stages in humans and the 2-cell stage in mice. EGA occurs in two phases: minor activation at male pronucleus of zygote and major activation at the 2-cell stage in mice (4). The functions of EGA are the degradation of oocyte specific transcripts, the replenishment of common transcripts by the embryo and gene expression of embryo specific transcripts. Most maternal RNA degradation starts upon oocyte maturation and ovulation and by the 2-cell stage about 70% are degraded (5). Though nuclear transcription ceases on oocyte maturation, poly(A) tails of existing transcripts are elongated leading to increased translation (6).

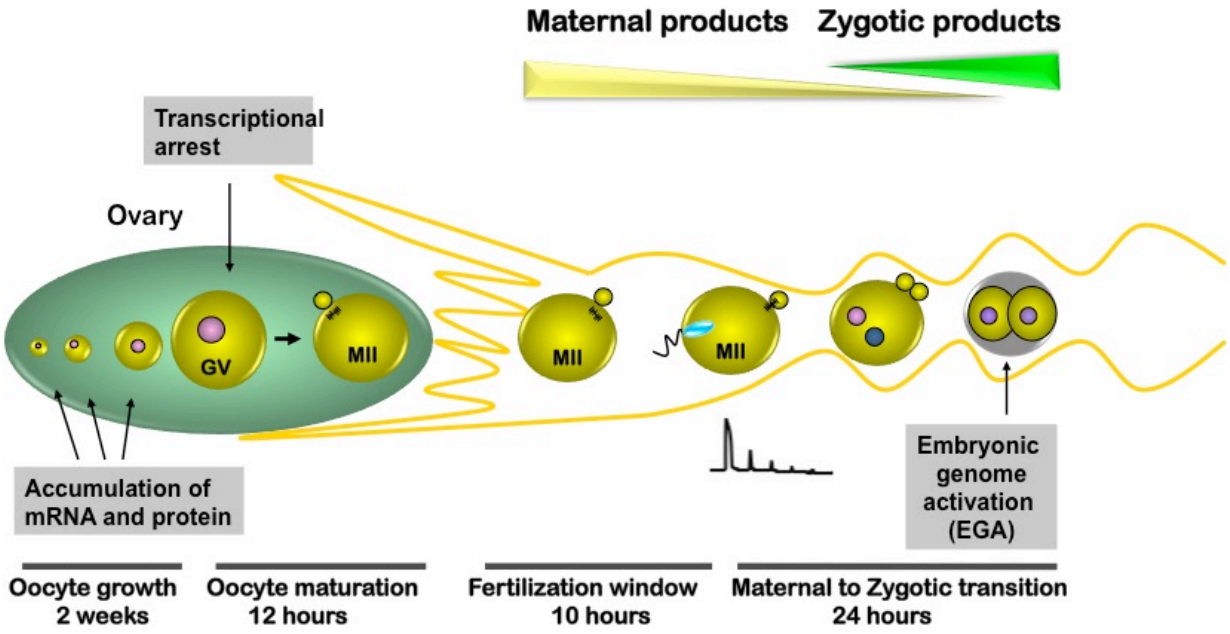

Figure 1.1. Overview of mouse oogenesis and egg to embryo transition

3. MATER (NLRP5) belongs to NLRP family

MATER (Maternal Antigen That Embryos Require) was originally identified as an autoantigen in the mouse model of autoimmune premature ovarian syndrome (7). MATER (official symbol: NLRP5) is a member of the NLRP gene family, which is a subfamily of CATERPILLAR family. The NLRP family is characterized by a pyrin domain (PYD), NACHT, and a leucine-rich repeat (LRR) domain (Fig. 2). N-terminal effector domain, PYD, has been found in proteins involved in inflammation and apoptosis, however, mouse NLRP5 has five tandem hydrophilic repeats (18 a.a) that has homology with dentrin matrix protein 1(DMP1) instead of the PYD (9). The central NACHT-NTPase domain containing ATP/GTPase-specific p-loop and Mg^{2+}-binding sites is found in proteins associated with innate immunity (8), and LRR domain is involved in protein-protein interaction.

There are 14 NLRPs in human and about 20 homologues in mice. NLRPs are known to have a role in apoptosis, inflammatory signaling pathways and reproduction. NLRP 1 and 3 act as the central scaffold of inflammasome during cell response to microbial infection. They activate caspase-1, which activate Interleukin-1B (IL-1β). NLRP3 is linked to five autoinflammatory syndromes as well (10). Regarding reproductive function of NLRPs, 12 isoforms of NLRPs are detected in human oocytes and preimplantation embryos and 7 isoforms in spermatozoa (11). Human NLRP7 is implicated in hydatidiform moles, female reproductive failure and testicular seminomas. Human NLRP14 is involved in preimplanatation development and spermatogenic failure (12-15). NLRPs expression levels decrease during maternal aging (15). It is possible that the mechanism of developmental failure of NLRPs could be related to inflammatory caspase activation with subsequent overproduction of IL-1β (16).

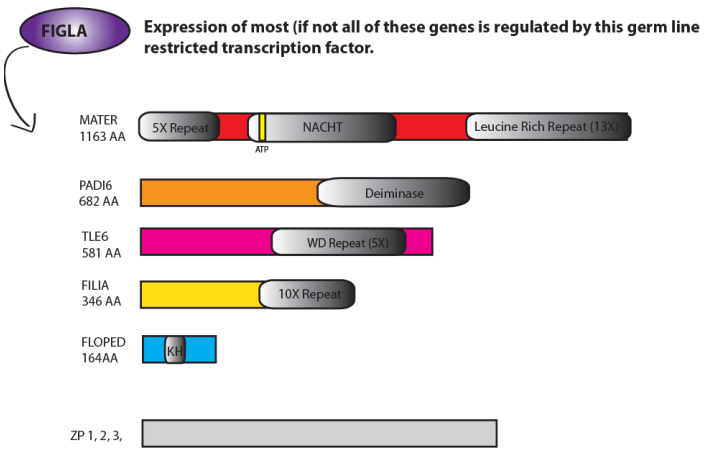

Figure 1.2. Components of SCMC (Adapted from Li et al. Dev Cell. 2008 Sep;15(3):416-25)

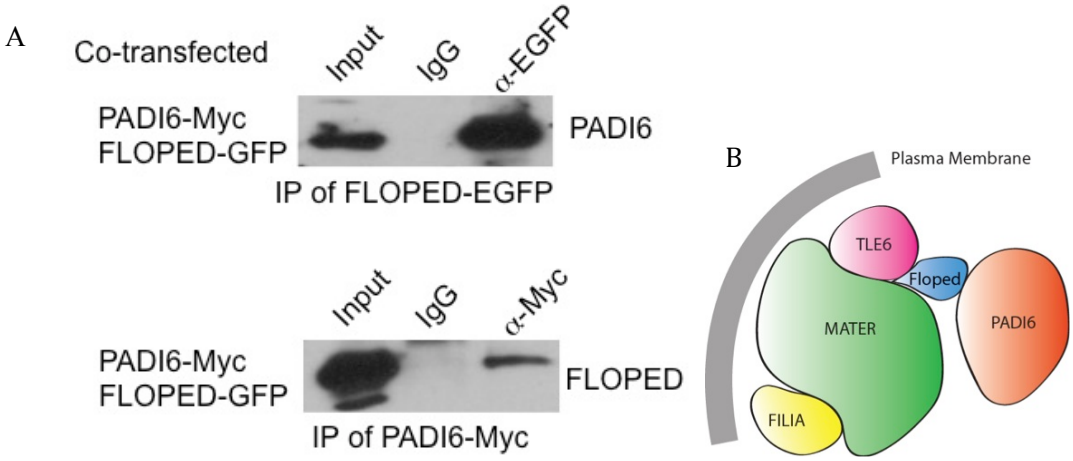

Figure 1.3. PADI6 is a putative component of SCMC

(A) Co-immunoprecipitation experiment of overexpressed PADI6 and FLOPED shows interaction of PADI6 with FLOPED in COS-1 cells. (B) SCMC model (Adapted from Li et al. Dev Cell. 2008 Sep;15(3):416-25)

4. *Mater*: a maternal effect gene that belongs to Subcortical maternal complex (SCMC)

MATER is a maternal effect gene product whose expression is restricted to oocytes and preimplanation embryos. The localization of MATER in oocytes is controversial in the literature: Tong et al. showed cytoplasmic localization, while Ohsugi et al. demonstrated cytocortical localization (17,18). Although the target sequences of antibodies are exactly same and both antibodies are made in rabbits, the difference of localization could be due to the method used to generate antibodies and the immunostaining techniques.

Mater$^{tm/tm}$ oocytes shows normal folliculogenesis, ovulation and fertilization, yet embryos conceived from *Mater*$^{tm/tm}$ oocytes arrest at the 2-cell stage. The *Mater*$^{tm/tm}$ phenotype is summarized in Table 1 (19). FILIA (daughter in Latin), another maternal factor, was identified as a binding partner of MATER (mother in Latin) (18). *Filia* null females show normal oogenesis, ovulation and fertilization, however, fertility is reduced due to abnormal chromosome stability and aneuploidy during early embryogenesis. FILIA warrants normal spindle assembly by regulating aurora kinase A (AURKA) and polo-like kinase 1 (PLK1), and normal spindle assembly checkpoint through MAD2 (20). Other maternal factors, Factor located in oocytes permitting embryonic development (FLOPED) and Transducin-like enhancer of split 6 (TLE6) have also been identified. FLOPED (Moep19) is a 15kD protein, which binds to RNA (21). TLE6, a mammalian homolog of Groucho, acts as a transcriptional corepressor in developmental processes (22). Double knockout mutants of *Mater* and *Floped* also showed a 2-cell arrest. FLOPED, TLE6, and MATER bind to each other and FILIA binds to MATER independently. FILIA, MATER, FLOPED and TLE constitute the SCMC (Fig. 3B). PADI6 was suggested as a putative binding partner of FLOPED based on the co-IP screening from *Floped*$^{+/+}$ and $^{-/-}$ oocytes, and co-immunoprecipitation analysis of overexpressed PADI6-Myc and FLOPED-GFP confirms

physical interaction of PADI6 and FLOPED suggesting PADI6 as another SCMC component (Fig. 3A). All SCMC components and PADI6 expression is excluded from the cell-cell contact regions from the 2-cell stage onwards and expression occurs only in the trophectoderm in blastocyst stage suggesting a possible role of SCMC in cell lineage establishment. FLOPED, TLE6, PADI6 and MATER are target genes of Factor in the germline alpha (FIGLA) based on microarray and serial analysis of gene expression (SAGE) (23). FIGLA is a basic helix-loop-helix (bHLH) transcription factor, which coordinates the expression of zona pellucida protein 1, 2, and 3. Females lacking FIGLA cannot form primordial follicles (Fig. 2) (24).

Table 1: Similarity of PADI6 and MATER

	PADI6	MATER
Tissue distribution	Unique to oocyte and preimplantation embryo	
RNA expression profile	Abundant in oocytes and mostly degraded by 2-cell embryo	
Protein expression profile	Primordial follicle to blastocyst (trophectoderm only)	Primary follicle to blastocyst (trophectoderm only)
Localization	Cytoplasm including CPLs and subcortex	
Upstream regulator	Target gene of Figla, a germ line specific transcription factor	
Regulation of CPL formation	Absence of CPL in *Padi6* null oocytes and 2-cell embryos	Severely reduced CPL in *Mater* $^{tm/tm}$ oocytes
phenotype of Padi6 null and *Mater* $^{tm/tm}$ mouse	Male fertile, Female infertilie ; folliculogenesis, ovulation, and fertilization normal but 2-cell arrest embryo	
Level of translation	Reduced de novo synthesis, however, spindlin is up-regulated in *Padi6* null and *Mater* $^{tm/tm}$ 2-cell embryo	
Level of EGA	47% of normal by TRC* synthesis 26% of normal by BrUTP in *Padi6* null 2-cell embryo	60% of normal by TRC* synthesis ~3% of normal by BrUTP in *Mater* $^{tm/tm}$ 2-cell embryo

*TRC: transcription requiring complex

5. Peptidylarginine deiminase 6 (PADI6): a member of the PADI family.

PADI6 is one isoform of the PADI family; PADIs are responsible for conversion of peptidylarginine into citrulline in a Ca^{2+} dependent manner (25,26). Citrulline is a non-coding native amino acid that is not integrated into protein upon translation, thus post-translational modification by PADIs produce peptidylcitrullines. Citrullination causes the loss of the positive charge, thus altering the protein structure, which could consequently facilitate changes in its function (27). There are five isotypes of PADI in mammals. Although one prokaryotic enzyme (AAF06719) can citrullinate both peptidylarginine and free L-arginine, it has no evolutionary link with mammalian PADIs (28). The five PADI paralogs share 45-55% amino acid sequence identity. The catalytic active site Cys 645 is located in the C-terminus where sequence identity is highly conserved among PADIs. PADI6 has about 40% sequence identity to the other PADI isoforms. However, PADI6 is unique in that it has an altered active cysteine site and a unique insertion site. Also, amino acid substitutions at Ca^{2+} binding sites raise the possibility that activity of PADI6 may not be dependent on Ca^{2+} (25). The main targets of PADIs are structural proteins. PADI1 is mainly expressed in the epidermis and citrullinates keratin and filaggrin (29). PADI2 is ubiquitously expressed and is abundant in the brain and in muscles. PADI3 is expressed in hair follicles and citrullinates tricohyalin (30). PADI4 is expressed in haematological systems and is a nuclear enzyme that citrullinates histones and acts as a transcriptional coregulator (26). PADI6 is exclusively expressed in oocytes and early embryos (31). Unlike other PADI members, the enzymatic activity of PADI6 is not clear and is under investigation.

6. Structures, components and regulation of CPLs

CPLs are unique, abundant structures present in mammalian oocytes. They are observed from the primary follicle to the blastocyst stage. The numbers of CPLs increase during oocyte maturation and are found to be abundant in preovulatory oocytes (32). They dynamically reorganize after fertilization, at compaction and at blastocyst formation. This reorganization is possibly associated with cell lineage determination (33). There are two types of CPLs; CPLs are solid and planar in rats and hamsters, and fibrous in humans, mice, dogs, cows and pigs (34). CPLs are also found in rabbits, sheep and monkey oocytes (35-37). Mouse CPLs are composed of 7-15 parallel 10-11nm filaments which are linked to each other by lateral cross-bridges spaced approximately every 25nm to form cylinders (Fig. 4). The surface of the filaments are masked by proteins with a mass of ~69kD (p69). The filaments were suggested as cytokeratins, however, the exact composition of CPLs remains unknown. Protein kinase M (PKM), the catalytic domain of Protein kinase C, was suggested as a regulator of CPLs, in that PKM remodels CPLs by binding and phosporylating cytokeratin and p69 upon fertilization.

7. Function of CPLs

Maternal ribosomes are stored in the eggs of most animals and contribute to translation after fertilization (38) and several lines of evidences proposed that CPLs play a role in storing ribosomes in mammalian oocytes. First of all, indirect evidence of the localization of ribosomes to CPLs comes from whole mount prepared mouse egg TEM images. CPLs give rise to periodic chains of particles with the approximate size of ribosomes. The ribosomes within the CPLs (21nm) are smaller and less electron-dense than free ribosomes (22.5nm) possibly due to interactions with proteins and an altered configuration (39). Second, CPLs are both RNase and Trypsin sensitive. Corroborating evidence of proteinaceous and RNA containing CPLs comes

from several fixation studies (40). Third, the precursor-product relationship between ribosomes and CPLs has been observed, whereas the number of ribosomes diminishes in the cytoplasm matrix, CPLs become abundant (41). Fourth, the actual number of ribosomes observed by TEM is ~80% less than theoretical ribosomes, calculated based on the amount rRNA, as CPLs become prominent (42). Lastly, about 75% of oocyte ribosomes can be sedimented at 9000g for 12 minutes, whereas somatic cell ribosomes can be pelleted after >1 hour centrifugation at 100,000g (6). To further develop the hypothesis that CPLs store ribosomes, 9000g sedimentation experiment was repeated using ribosomal component S6 antibody in *Padi6* WT and null oocytes. The idea was that if ribosomes are stored in a large CPL complex, large portions of S6 will sediment more rapidly in *Padi6* WT oocytes. However, S6 in *Padi6* null oocytes should pellet less readily. Indeed, majority of S6 partitioned in the supernatant of *Padi6* null oocytes as opposed to *Padi6* WT oocytes (43). To date, lattice-like ribosome storage sites were described in humans, protozoans, chickens and lizards (44-46).

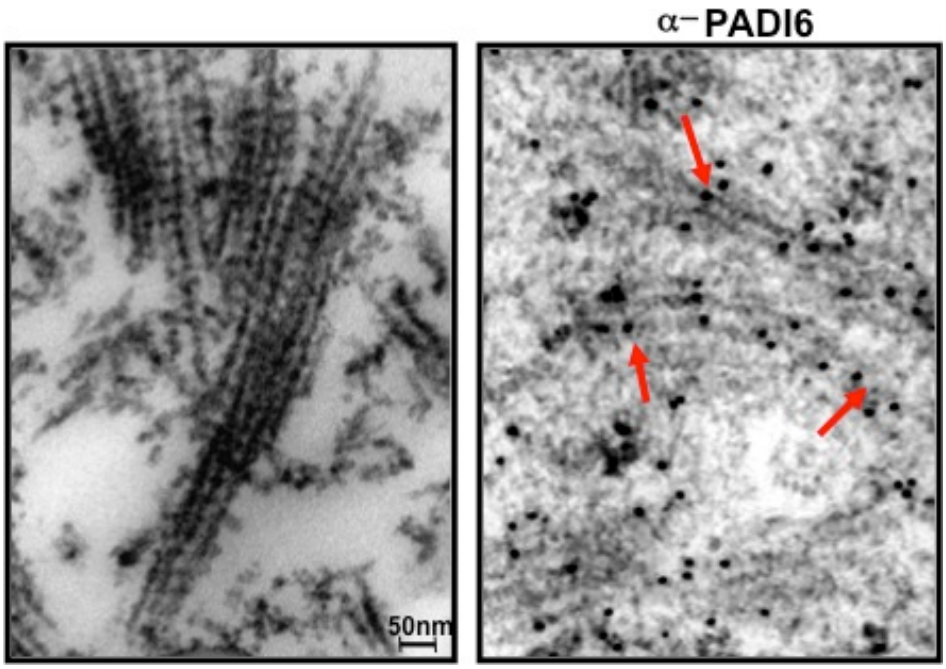

Figure 1.4. The structure of Cytoplasmic lattices (CPLs) and predominant localization of PADI6 to CPLs (Wright et al., Dev.Bio. 2003)

8. The phenotype of *Padi6* null mice

PADI6 is expressed from the primordial stage and persists up to the blastocyst stage; most mRNA is degraded upon oocyte maturation. PADI6 localizes to the cytoplasm, the cortex and mainly to CPLs by IEM (4) (31). *Padi6* null males are fertile while *Padi6* null females are infertile. Although folliculogenesis, ovulation and fertilization seem normal in the *Padi6* null oocytes, embryos from *Padi6* null females are unable to progress beyond the 2-cell stage. Interestingly, in *Padi6* null oocytes, CPLs do not form indicating that PADI6 is required for CPL formation (47). While global levels of ribosomal subunit protein S6 and 18S rRNA are not affected in *Padi6* null oocytes, solubility of S6 increases as seen by confocal microscopy and oocyte fractionation. Unlike *Padi6* null oocytes, the global level of S6 is decreased and the cortical localization of S6 is disrupted in *Padi6* 2-cell embryos leading to defective protein synthesis. This altered translation results in defective EGA. The *Padi6* null phenotype and similarity of MATER and PADI6 is summarized in Table 1 (43).

9. Significance of study

Infertility affects about 15% of the population of the United States (48). In spite of increased understanding of the causes of infertility, ~10% of female infertility cases remain unidentified (49). Assisted Reproductive Technologies (ARTs) have led to increased success of fertilization; however despite this increased success, one of the major problems in *in vitro* fertilization (IVF) clinics remains the low rate of successful pregnancy. Genetic defect-related embryo deaths and issues with implantation appear to result in the difference between fertilization and pregnancy rates (80% versus 30%) (50). Thus women with a defect in a maternal effect gene, which can result in genetic defect-related embryo death, are anticipated to contribute to the unexplained

infertility cases discussed above. The term "maternal effect" refers to the phenotype of the embryo which is dependent on the genotype of the mother. The characterization of the molecular mechanism of maternal effect genes will shed new light on genetic diagnoses and the treatment of oocyte-based female infertility. I propose to investigate the molecular mechanisms of 2-cell arrest in *Mater*$^{tm/tm}$ embryos. I will use a mouse model to elucidate the role of MATER (46% sequence identity with human MATER) in female infertility. The proposed study will contribute to understanding genetic defects underlying idiopathic infertility and has the potential to lead to the development of infertility therapies.

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CHAPTER II

Role for MATER in Cytoplasmic Lattice Formation in Murine Oocytes

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Abstract

Background: *Mater* and *Padi6* are maternal effect genes that are first expressed during oocyte growth and are required for embryonic development beyond the two-cell stage in the mouse. We have recently found that PADI6 localizes to, and is required for the formation of, abundant fibrillar Triton X-100 (Triton) insoluble structures termed the oocyte cytoplasmic lattices (CPLs). Given their similar expression profiles and mutant mouse phenotypes, we have been testing the hypothesis that MATER also plays a role in CPL formation and/or function.

Methodology/Findings: Herein we show that PADI6 and MATER co-localize throughout the oocyte cytoplasm following Triton extraction, suggesting that MATER co-localizes with PADI6 at the CPLs. Additionally, the solubility of PADI6 was dramatically increased in $Mater^{tm/tm}$ oocytes following Triton extraction, suggesting that MATER is involved in CPL nucleation. This prediction is supported by transmission electron microscopic analysis of $Mater^{+/+}$ and $Mater^{tm/tm}$ germinal vesicle stage oocytes which illustrated that volume fraction of CPLs was reduced by 90% in $Mater^{tm/tm}$ oocytes compared to $Mater^{+/+}$ oocytes.

Conclusions: Taken together, these results suggest that, similar to PADI6, MATER is also required for CPL formation. Given that PADI6 and MATER are essential for female fertility, these results not only strengthen the hypothesis that the lattices play a critical role in mediating events during the oocyte-to-embryo transition but also increase our understanding of the molecular nature of the CPLs.

Introduction

A unique feature of mammalian oocytes is that transcription ceases upon oocyte maturation (1) and does not resume until embryonic transcription becomes activated in the early embryo (2-5). During this period of transcriptional quiescence, the oocyte must rely on maternal factors, structures, and organelles that have accumulated in the oocyte during growth to mediate this critical period, often called the oocyte-to-embryo transition (OET). In non-mammalian species, mutation analysis has identified a large number of factors, called maternal effect genes (MEGs), which are synthesized and accumulate in the oocyte and then persist in the early embryo where they are required for embryonic development (6,7). Phenotypic analysis of mouse knockout models has recently lead to the identification of several mammalian MEGs such as Maternal Antigen That Embryos Require (MATER) and Peptidylarginine Deiminase 6 (PADI6), two highly-abundant oocyte-restricted proteins that are essential for embryonic development beyond the two-cell stage (8,9).

MATER (gene name, NLRP5) was originally identified as an antigen that is involved in a mouse autoimmune oophoritis (10). Recently, MATER (mother in Latin) has been identified as a component of the SCMC (subcortical maternal complex) along with other maternal factors including FILIA (daughter in Latin), FLOPED, and TLE6 (11). Additionally, PADI6 has also been putatively identified as a component of the SCMC complex. While FILIA is thought to play a role in chromosome stability during embryogenesis (12), the role of MATER remains to be elucidated.

PADI6 was originally cloned from the mouse oocyte proteome due to its abundance in metaphase II-arrested oocytes and its oocyte-restricted expression pattern (9). Interestingly, PADI6 is localized to, and required for, the formation of an abundant, oocyte- and early embryo-

restricted structure, the cytoplasmic lattices (CPLs or lattices) (9,13). The lattices are composed of 5-7 parallel fibers with each fiber containing a repeating unit of ~20nm (14). The bundled fibers are first observed at early stages of oocyte growth (30-40μm) (15) and persist in the early embryo until the blastocyst stage (16). CPLs were found to be resistant to Triton-X-100 (Triton), thus, extraction with this detergent provides a valuable tool for studying CPL associated proteins (14,17). While CPLs have been observed by electron microscopy since the 1960s, their function remains poorly understood. Based on electron microscopy and biochemical analysis, a number of older reports predicted that the lattices may function as yolk granules (18) or as a ribosomal storage site (15,19-23), with the latter hypothesis being supported by recent data from our lab (24).

Interestingly, *Padi6* and *Mater* share many similar properties. For example, the expression of both maternal genes is regulated by the basic helix-loop-helix transcription factor, FIGLA (Factor in the germline alpha) (25) and is restricted to oocytes and early embryos in mouse. Microarray analysis (26) along with previous studies (9,27,28) suggest that both transcripts appear in the oocyte at the primordial/primary follicle stage and then abruptly disappear around meiotic maturation. MATER and PADI6 protein expression roughly parallels that of their transcripts in oocytes; however, protein levels persist at high levels throughout preimplantation development until the blastocyst stage (9,27). Additionally, analysis of *Padi6*$^{-/-}$ and *Mater*$^{tm/tm}$ (tm: targeted mutation) female mice indicates that the phenotypes of embryos conceived from these two mutants are strikingly similar with a developmental arrest occurring at the two-cell stage; likely due to abnormal embryonic genome activation (EGA) as demonstrated by reduced levels of BrUTP and TRC transcripts in these embryos (8,13,24).

Based on these similarities, we hypothesized that, similar to PADI6, MATER may also play a role in CPL formation. Here we show that PADI6 and MATER co-localize throughout the oocyte cytoplasm following Triton extraction and appear to both be associated with large complexes of similar molecular weight. Additionally, the solubility of PADI6 (a CPL marker) is greatly increased in *Mater* hypomorph oocytes, suggesting that lattices are significantly reduced in *Mater*$^{tm/tm}$ oocytes. As a more direct confirmation of the requirement of MATER for CPL formation, we show by electron microscopy that the volume of CPLs is reduced 9.65-fold in germinal vesicle (GV) stage *Mater*$^{tm/tm}$ oocytes. Taken together, these results suggest that MATER is required for CPL formation. Studies are currently underway to test the hypothesis that MATER's role in CPL synthesis is due to direct interactions with other CPL-associated factors and to further investigate CPL function using the *Mater*$^{tm/tm}$ mouse oocyte model.

Results

PADI6 and MATER co-localize in oocytes and early embryos and appear to associate with high molecular weight complexes.

The localization of MATER and PADI6 has been reported previously (9,27,28). Given that PADI6 primarily localizes to the CPLs (9) and is required for CPL formation (13), we believe that PADI6 represents a good marker for the CPLs. Therefore, to begin testing the hypothesis that MATER plays a role in CPL function, we first carried out confocal immunofluorescence microscopy (CIM) to determine if these two maternal factors co-localize. Oocytes and embryos were fixed with paraformaldehyde (PFA), permeabilized and stained with anti-PADI6 and anti-MATER antibodies. The results showed that MATER and PADI6 appear to strongly co-localize in the cortex and, to a lesser degree, in the cytoplasm of GV stage oocytes. Co-localization was

also observed at the non-opposed cortex regions of 2-cell and 4-cell stage embryos with less co-localization being observed throughout the cytoplasm in the early embryos (Fig. 1A). Interestingly, in two and four cell embryos, PADI6 staining is more strongly localized to the cortex than MATER, which can be seen to penetrate deeper into the cytoplasm. CPLs are Triton insoluble structures. Therefore, to more directly test the hypothesis that MATER localizes to the CPLs, we next extracted GV oocytes with 0.1% Triton for 10 min and then carried out co-localization analysis using CIM. Following Triton extraction, the cytoplasmic signal of MATER and PADI6 was greatly enhanced throughout the oocyte cytoplasm, thus providing an indirect line of evidence that MATER is associated with the CPLs (Fig. 1B). To test if MATER and PADI6 co-localize in the extracted oocytes, co-localization analysis was performed with Zeiss 2007 software and the scatterplot shows the degree of co-localization between MATER and PADI6 in the extracted oocytes. Mander's overlap and Pearson's correlation coefficients (0.97 and 0.7, respectively) confirm the high degree of colocalization between MATER and PADI6 (Fig. 1B).

To further strengthen the potential association between MATER and PADI6, we next resolved proteins from GV stage outbred CD1 mouse oocyte extracts using fast protein liquid chromatography (FPLC) and eluted fractions were analyzed by immunoblot using MATER and PADI6 antibodies. Results show that PADI6 and MATER appear to co-elute in three different fractions. An initial high molecular weight fraction is seen between 2000kD and 669kD, a second fraction is observed between 669 and 220 kD, and a third peak is also seen that is below the 200 kD molecular weight marker. Additionally, a fraction of PADI6 also appears to elute independent from MATER just below the 200 kD marker. The observation that PADI6 and

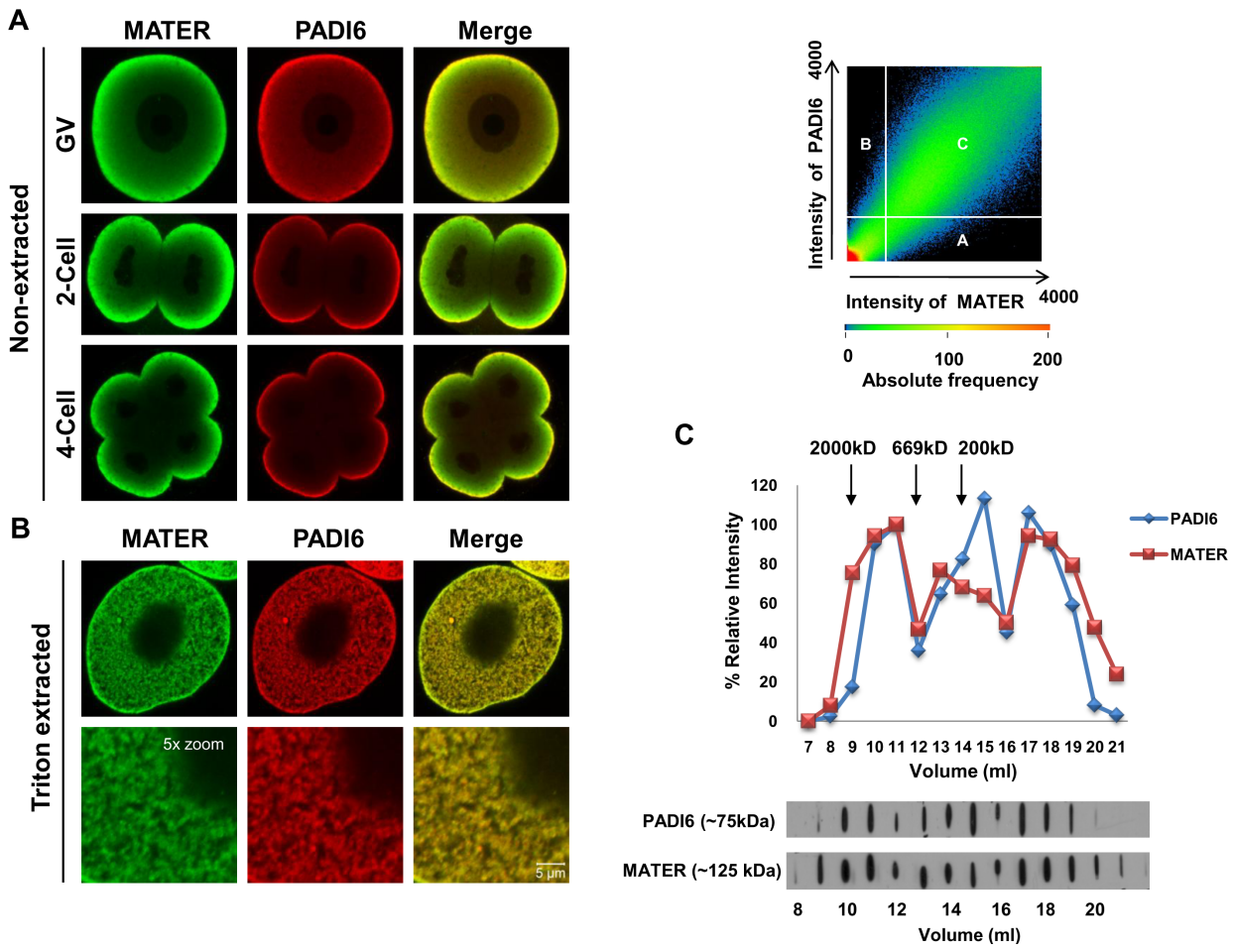


Figure 2.1. Co-localization of MATER and PADI6 in non-extracted and Triton X-100 extracted GV oocytes and early embryos

(A) Confocal microscopic images show non-extracted GV-stage oocytes, 2-cell, and 4-cell embryos after fixation, permeabilization and incubation with anti-MATER (green) and anti-PADI6 (red) antibodies. **(B)** GV oocytes extracted with 0.1% Triton X-100 and stained with MATER (green) and PADI6 (red) antibodies. Merged images highlight MATER and PADI6 co-localization. Scatter plot indicates a degree of co-localization of MATER and PADI6. Region A shows pixels with high MATER (green) intensities, region B shows pixels with high PADI6 (red) intensities, and region C shows pixels with both high MATER (green) and PADI6 (red) intensities. Mander's overlap coefficient: 0.97, Pearson's correlation coefficient: 0.7. **(C)** CD1

mouse oocyte lysates were chromatographed by FPLC. Eluted fractions (1ml) were analysed by immunoblotting with antibodies to PADI6 and MATER. Densitometry was used to generate a graph and the values in fraction 11 were set at a relative intensity of 100%. Elution of each protein standard is indicated by arrow.

MATER appear to co-elute in high (~1,000 to 2,000) molecular weight fractions supports the hypothesis that these two maternal proteins are associated with a supramolecular complex (Fig. 1C).

The Triton X-100 solubility of PADI6 is increased in *Mater*$^{tm/tm}$ oocytes.

The oocyte CPLs cannot be visualized by EM in *Padi6* null mouse oocytes (24), suggesting that the CPLs do not form in the absence of PADI6 and that proteins which normally associate with the CPLs are rendered more soluble in the mutant eggs. This idea is supported by our previous finding that the Triton solubility of ribosomal components, such as S6, is greatly enhanced in *Padi6*-null eggs (24). Therefore, we decided to use a similar type of approach in *Mater* mutant oocytes to more directly test the hypothesis that MATER plays a role in CPL formation and function. We predicted that if CPLs do not form in *Mater*$^{tm/tm}$ oocytes, then PADI6 (a CPL-associated protein) should become more soluble following Triton extraction. GV oocytes were either not extracted or extracted with 0.1% Triton for 10 minutes and prepared for CIM as described above. Results showed that, in non-extracted oocytes, the level and localization of PADI6 was similar between *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes with slightly reduced PADI6 level at the cortex in *Mater*$^{tm/tm}$ oocytes. However, PADI6 levels were dramatically reduced in *Mater*$^{tm/tm}$ oocytes following Triton extraction (Fig. 2A). In order to better quantitate these findings, we carried out western blot analysis of non-extracted and Triton extracted oocytes. In line with our confocal findings, immunoblot analysis showed that PADI6 levels were similar between wild type and *Mater* hypomorph oocytes. However, following Triton extraction, analysis of the insoluble fraction found that, while levels of β-actin were similar, image densitometry quantification revealed that levels of PADI6 were reduced by ~50% in the mutant

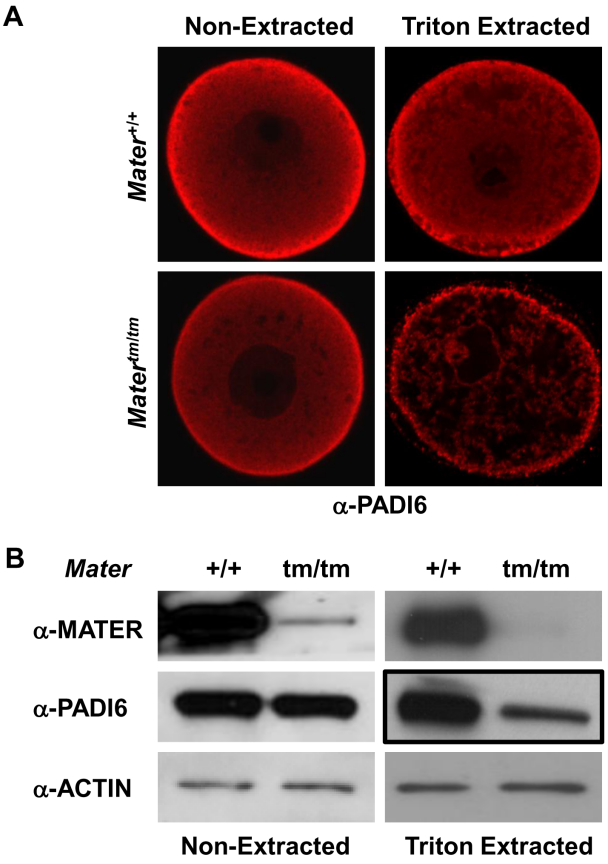


Figure 2.2. PADI6 Triton X-100 solubility is increased in *Mater*$^{tm/tm}$ GV-stage oocytes.

(A) Confocal analysis shows *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ GV-stage oocytes prior to, and following, extraction with 0.1% Triton X-100. Oocytes were incubated with PADI6 antibodies (red). **(B)** Western blotting shows expression of MATER and PADI6 protein in *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ GV-stage oocytes prior to, and following, 0.1% Triton X-100 extraction. Isolated oocytes were either extracted or not extracted with Triton, and then evaluated by Western blotting using either anti-MATER, anti-PADI6, or anti-β actin antibodies.

compared to the control wild type oocytes (Fig. 2B). Taken together, these results suggest that the lattices do not form (or form to a lesser degree) in the absence of MATER, thus releasing PADI6 from the insoluble fraction.

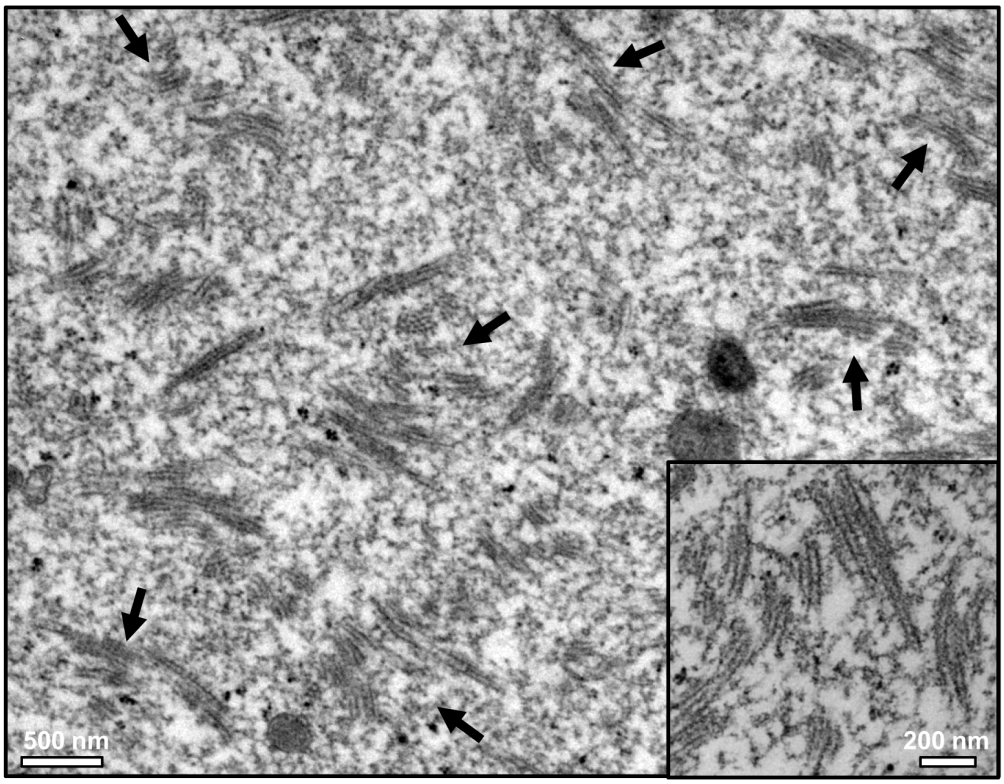
Morphometric electron microscopic analysis reveals that CPL volume is greatly reduced in M*ater*$^{tm/tm}$ oocytes.

In order to directly test the hypothesis that MATER is required for CPL formation, we next performed morphometric analysis of the CPLs in *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes using transmission electron microscopy (TEM). GV oocytes were collected from three *Mater*$^{+/+}$ and three *Mater*$^{tm/tm}$ female mice, fixed, embedded with resin, and ultrathin sections of the blocks were placed on a grid. The oocytes were then imaged using TEM and quantitation of the CPLs and other organelles was then carried out using the point-counting method (29). Results showed that the average volume fraction of CPLs per area was 5.5% for *Mater*$^{+/+}$ oocytes and 0.057% for *Mater*$^{tm/tm}$ oocytes, thus the volume fraction of CPLs in *Mater*$^{tm/tm}$ oocytes was reduced by 9.56 fold when compared to *Mater*$^{+/+}$ oocytes. Standard error of mean (SEM) of the CPL volume fraction for *Mater*$^{+/+}$ oocytes was 0.0035 and for *Mater*$^{tm/tm}$ oocytes was 0.0009 (Fig. 3) (Fig. 4C). This result provides direct support of our hypothesis that MATER is required for CPL formation. It is important to note that a residual amount of MATER protein is present in *Mater*$^{tm/tm}$ oocytes (See Fig. 2B and Discussion below), thus possibly explaining why some lattices are observed in hypomorph oocytes.

Interestingly, while scoring CPL volume by TEM, we also noticed that there was an apparent increase in the volume of lipid droplets in *Mater*$^{tm/tm}$ oocytes when compared to *Mater*$^{+/+}$ oocytes (Fig. 4A). To quantify this observation, we then carried out morphometric

A

Mater$^{+/+}$



B

Mater$^{tm/tm}$

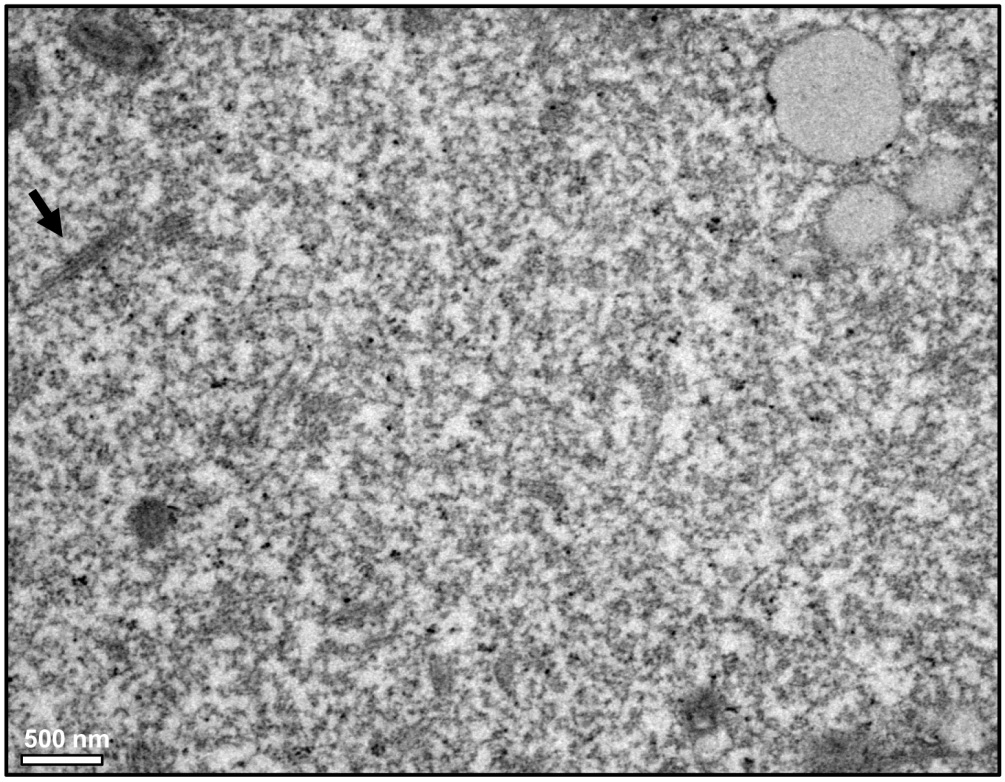


Figure 2.3. EM analysis reveals that the volume fraction of CPLs is reduced in *Mater*$^{tm/tm}$ oocytes.

(A) Representative TEM image (x11,500) of the ultrastructure of *Mater*$^{+/+}$ GV oocytes. A high magnification (x26,500) image of the lattice structure is shown in the inset. **(B)** Representative TEM image (x11,500) showing the ultrastructure of *Mater*$^{tm/tm}$ GV oocytes. Arrows in **A** and **B** indicate CPLs.

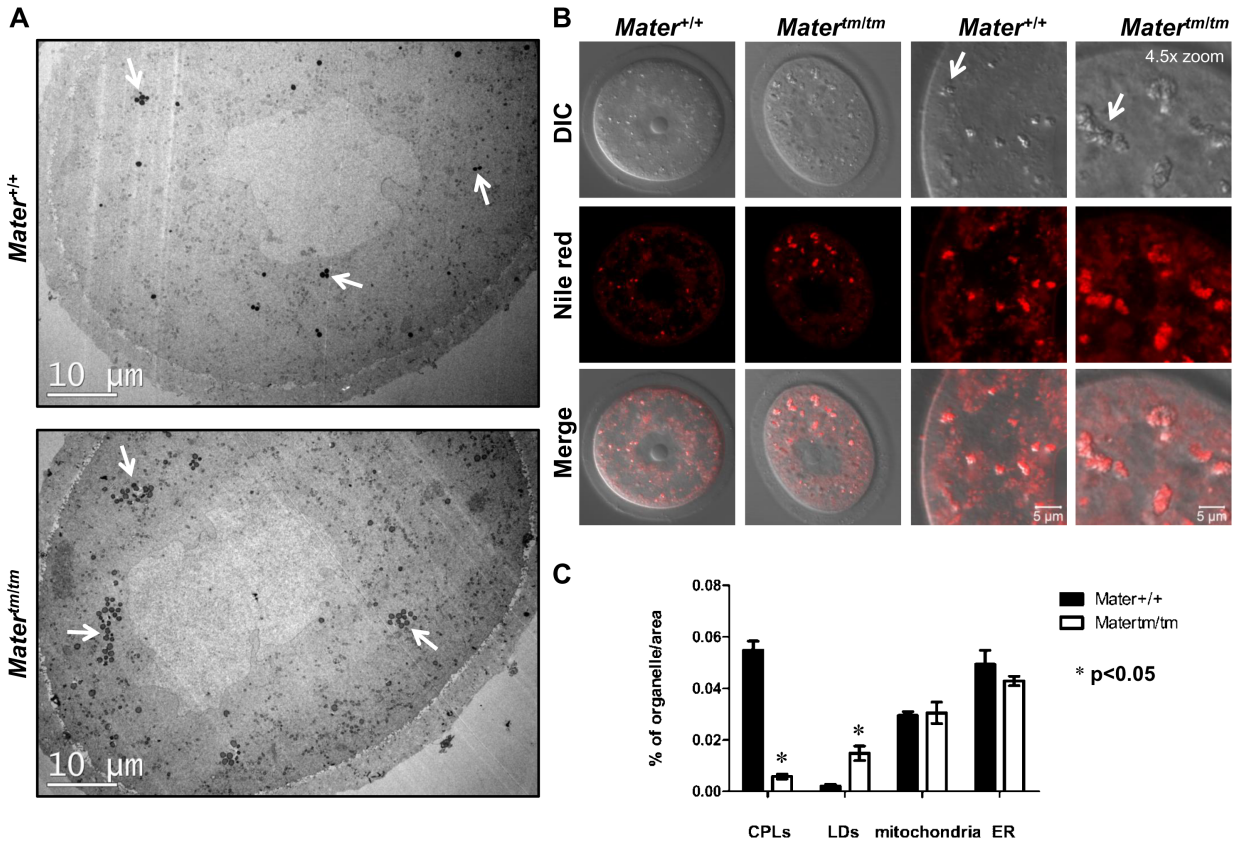


Figure 2.4. *Mater*$^{tm/tm}$ oocytes display elevated levels of lipid droplets.

(A) Low magnification (x1,700) TEM images of *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ GV-stage oocytes. Oocytes were prepared for TEM as above. White arrows point to LDs. **(B)** Confocal images of GV-stage live *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes following Nile red staining. DIC images show morphology of LDs in oocytes. Close up images (Right panel) highlight LD aggregates. Arrows indicate LDs. **(C)** Quantitation of the volume fractions of CPLs, LDs, mitochondria, and ER in *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ GV-stage oocytes. Mean ± SEM is indicated. *P* value is < 0.004 for CPLs, <0.04 for LD, > 0.05 for mitochondria, and ER.

analysis of the LDs in the wild type and mutant oocytes. Additionally, we also scored mitochondrial and ER volume to investigate if the volume of these organelles might be altered in the *Mater*$^{tm/tm}$ oocytes. We found that, while the volume fractions of mitochondria and ER were similar, the volume of lipid droplets increased approximately 7-fold in *Mater*$^{tm/tm}$ oocytes (Fig. 4C). The finding that mitochondrial volume fraction did not appear to be affected in *Mater*$^{tm/tm}$ oocytes is interesting in light of previous immuno-EM data showing that MATER localizes, in part, to mitochondria. Experiments are being carried out to address this observation. In support of our EM findings on the increase in lipid droplet volume fraction in *Mater*$^{tm/tm}$ oocytes, we also noticed that the morphology of these oocytes seems distinct from *Mater*$^{+/+}$ oocytes in that brown aggregates were observed in *Mater*$^{tm/tm}$ oocytes under the dissecting microscope. To test if these aggregates were LDs, we stained live *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes with Nile red and the merged images show that the aggregates are, in fact, LDs. (Fig. 4B). Finally, while not quantitated, we also noticed that the LDs also tended to form larger clusters in *Mater*$^{tm/tm}$ oocytes. Quantitation of the CPLs and organelles is shown in Figure 4C.

Discussion

In this manuscript, we define a new role for MATER by showing that this maternal effect gene product appears to be required for CPL formation. Our EM morphometric analysis of the volume of CPLs in wild type and *Mater*$^{tm/tm}$ oocytes provides the most direct support of this conclusion with results showing that the volume fraction of CPLs is reduced by ~10-fold in *Mater*$^{tm/tm}$ oocytes when compared to wild type oocytes. It is important to note here that the *Mater*$^{tm/tm}$ mice used in this study were derived from the same mice that were originally characterized as *Mater* null by Tong et al (8). Subsequently, however, Ohsugi et al (28) later documented that these

mice actually express low levels of MATER protein, and thus termed the mice *Mater*$^{tm/tm}$ or hypomorphs. We confirmed that these mice do express low levels of MATER protein and thus used the *Mater*$^{tm/tm}$ hypomorph terminology for this report. Interestingly, in our study, the observed reduction in CPL volume roughly correlates with the 90% reduction in MATER protein in the *Mater*$^{tm/tm}$ oocytes, suggesting that there may be a direct 1:1 stoichiometry between MATER and CPLs. In other words, decreasing MATER protein concentrations would result in a corresponding decrease in lattice number. Thus, by extrapolation, we predict that lattices would be absent from true *Mater* null oocytes.

With respect to the role of MATER in lattice formation, while it is possible that MATER functions indirectly (as an upstream signaling factor for CPL formation, for example), we predict that MATER's role in lattice formation is direct via associations with other CPL components. This prediction is based on our observation that PADI6 (a *bona fide* CPL component) and MATER co-localize with each other throughout the cytoplasm and on Tashiro's immuno-EM finding that MATER localizes to the CPLs (30). Therefore, MATER appears to represent a component of the CPL superstructure. Further, the observation that PADI6 solubility is increased in *Mater*$^{tm/tm}$ oocytes suggests that MATER plays a direct role in helping to nucleate CPL-associated proteins into the mature CPL superstructure and that, in the absence of MATER, CPL-associated proteins are rendered more soluble.

As noted in the introduction, MATER has also recently been found to be associated with, and required for, the formation of another supramolecular complex, termed the SCMC, which also contains FILIA, TLE6, and FLOPED. Further investigation into the molecular nature of this complex finds that *Mater*, *Filia*, and *Floped*-null mice are essential for development beyond the early cleavage stages of development. At the molecular level, FPLC and immunoprecipitation

analysis suggests that the SCMC is formed by a direct interaction between FLOPED, MATER and TLE6, while FILIA appears to associate with the SCMC via direct interaction with MATER alone. At the subcellular level, these proteins appear to co-localize at the oocyte subcortex and this localization becomes asymmetrically restricted to apical cytocortex of two-cell embryos. In morulae, however, expression of the SCMC proteins is limited to the non-opposed cytocortex of outer blastomeres and these proteins are not observed within the inner cells. This restricted localization pattern has lead to the hypothesis that the SCMC structure may provide a molecular marker of embryonic cell lineages and possibly cell fate determinations (11). While the role of the SCMC in early development remains to be elucidated, analysis of *Filia*-null mice suggests that this maternal factor plays an important role in integrating the spatiotemporal localization of regulators of euploidy and cell cycle progression during early development (12).

Interestingly, a very recent report by another group (30) has further characterized *Floped*-null mouse oocytes by electron microscopy and found that CPLs are also absent from these mutant oocytes, thus indicating that this SCMC protein is also required for lattice formation. Additionally, they found that, while confocal immunofluorescence (IF) analysis of isolated oocytes/embryos suggested that FLOPED localized to the cytocortex as shown previously (11), immuno-EM analysis indicated that FLOPED primarily localized at the CPLs throughout the cytoplasm. The investigators then predicted that this conflict in FLOPED subcellular localization patterns was due to the inability of anti-FLOPED antibodies to penetrate the cortex of isolated oocytes/embryos, thus resulting in a strong cortical FLOPED staining pattern. They then tested this prediction by staining paraffin embedded cross-sections of oocytes and embryos and found that, under these conditions, FLOPED staining was seen throughout the

cytoplasm and was not concentrated at the cortex. Taken together, the findings by Tashiro et al. indicate that FLOPED primarily localizes to the CPLs and is also required for lattice formation.

In this report, we first document the co-localization of MATER and PADI6 at the cortex of non-extracted oocytes/embryos, and throughout the cytoplasm of Triton extracted oocytes. We predict that the punctate co-localization of PADI6 and MATER throughout the cytoplasm of Triton extracted oocytes is reflective of the localization of these maternal factors to the Triton-resistant CPLs. This prediction is supported by our previous immuno-EM finding showing that PADI6 primarily localizes to the CPLs and by the new Tashiro publication (30) which shows that, anti-MATER coated gold particles also localize to the CPLs. While we currently do not fully understand why PADI6 and MATER confocal IF staining is primarily limited to the cortex in non-extracted oocytes, the new findings on FLOPED localization by Tashiro raise the possibility that the observed cortical localization of PADI6 and MATER is artifactual in nature. In fact, we have found that the ratio of cortical versus cytoplasmic PADI6 and MATER staining in intact oocytes can vary depending on the fixation and immunostaining conditions used (27,28). Alternatively, it is also possible that MATER and PADI6 are associated with the SCMC at the cortex and with the CPLs throughout the cytoplasm. The hypothesis that PADI6 is associated with the SCMC is supported by previous work showing that PADI6 potentially associates with FLOPED, a component of the SCMC (11). If this hypothesis is correct, then it is possible that the SCMC and CPL complexes are structurally related and may exist as a continuum that cannot be resolved by electron microscopy. Thus, PADI6, MATER, and likely other proteins could be found to shuttle between the CPLs and the SCMC. Interestingly, our FPLC data may provide some support for this prediction. Our analysis shows that PADI6 and MATER appear to co-elute in three separate fractions. The initial high molecular weight fraction

(~670 to 2,000) is similar in mass to that shown for MATER and other SCMC components in the previous report (11). This finding supports the hypothesis that PADI6 is a component of the SCMC. In our study, the observed midrange ~200 to 670 kDa co-elution peak raises the possibility that MATER and PADI6 may also be associated with a smaller complex that either may not be directly associated with a specific structure. We note that the lowest molecular weight MATER and PADI6 co-elution peaks are well below the 200 kDa mass marker and predict these fractions represent monomeric forms of PADI6 and MATER. While it is currently unclear why Li et al did not also observe the midrange and lower MW peaks for MATER in their study, a comparison of protocols finds that we utilized different sized columns which may have affected protein resolution. Additionally, we also added 0.1% Trition to our oocyte lysis buffer, which may have affected protein solubility. It currently remains unclear whether the association between PADI6 and MATER is direct or indirect. For this study, we attempted numerous co-immunoprecipitation experiments and were not able to show an interaction between PADI6 and MATER. Interestingly, given that PADI6 was identified as a potential interacting partner with FLOPED and that FLOPED directly interacts with MATER, it is possible that PADI6 and MATER indirectly associate via interactions with FLOPED.

An unexpected finding from this study was that, in addition to the reduction of CPLs in *Mater*$^{tm/tm}$ oocytes, the volume of lipid droplets was significantly increased in the mutant oocytes. LDs are dynamic organelles that are primarily thought to store energy in the form of neutral lipids such as triacylglycerols and sterol esters (31). In spite of the significant progress in LD research in recent years, the fundamental mechanisms by which LDs function remain mostly unknown. This is particularly true for mammalian oocytes, as there are only a few reports suggesting a role for LDs in oocyte function (32-35). Interestingly, LD accumulation is

frequently seen in pathologic conditions such as apoptosis, cancer, and inflammation (36). A recent report has found an interaction between MATER and PKC {epsilon}, which is involved in the anti-apoptotic pathway (36,37). If MATER is indeed associated with an anti-apoptotic signaling pathway, LD accumulation in *Mater*$^{tm/tm}$ oocytes may be associated with apoptotic events. Importantly, our findings indicate that *Mater*$^{tm/tm}$ mice represent a valuable tool to study LD function in oocytes.

To conclude, in this study we show that MATER is required for CPL formation and also appears to represent a component of the CPL superstructure. In light of the new finding by Tashiro that another SCMC component, i.e. FLOPED, is also required for CPL formation, and that PADI6 was identified at a putative FLOPED interacting proteins, our new findings now highlight a potential relationship between the SCMC and the CPLs. Additionally, given the oocyte/embryo-restricted nature of PADI6 and MATER and that these factors are all essential for early cleavage divisions, these findings further highlight the importance of the CPLs in mediating the oocyte-to-embryo transition. Furthermore, given that MATER and PADI6 are both expressed in human oocytes, a better understanding of the role of murine MATER, PADI6, and the lattices in early developmental events may provide insight into human infertility.

Materials and Methods

Ethics statement

Animals were bred and maintained in accordance with Cornell animal care guidelines and animal protocol number 2007-0113 was approved by the Cornell Institutional Animal Care and Use Committee before implementation.

Animals

The generation and validation of the *Mater*$^{tm/tm}$ mouse strain has been described previously (8). CD-1 breeding mice were purchased from commercial vendors.

Collection and culture of oocytes and embryos

Oocytes were harvested from 4- to 6-week-old *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ mice. GV-stage oocytes were harvested 46-48 hours after intraperitoneal injection of 5IU pregnant mare serum gonadotrophin (PMSG). 46-48hours post PMSG, 5IU of human chorionic gonadotrophin (hCG) was administered intraperitonealy and Metaphase II (MII) oocytes were harvested 12-14 hours later. Oocytes were collected in M2 medium (Sigma) and 5 μM milrinone (Sigma) or 200 μM IBMX (Sigma) was added to the media when GV oocytes were used. For collection of preimplantation embryos, mice were superovulated with PMSG/hCG as above, mated, and two-cell and four-cell embryos were collected 2 and 3 days later, respectively.

Confocal microscopy

Immunostaining and Triton extraction procedures for oocytes have been described elsewhere (Yurttas et al., 2008). Oocytes and embryos were fixed in 4% paraformaldedyde (PFA) (EM Sciences) for 30 minutes. For extraction, oocytes were incubated in extraction buffer containing 0.1M KCl, 20mM $MgCl_2$, 3mM EGTA, 20 mM HEPES (pH 6.8), 0.1% Triton X-100 and 1x Complete Protease Inhibitor Cocktail (Roche) for 10 minutes and rinsed in PBS quickly and fixed. Oocytes were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes, washed and incubated with rabbit anti-MATER (1:1000) (Tong et al., 2004) or guinea pig anti-PADI6

(1:1000) (Wright et al., 2003) in IF buffer (1% BSA, 0.5% Normal Goat Serum in PBS) for 1 hour followed by another 1h incubation with the appropriate Alexa Fluor-conjugated (1:450) secondary antibody (Molecular Probes) in IF buffer. Oocytes were mounted on slides with Slowfade Gold antifade agent (Molecular Probes) and imaged using an LSM 510 laser scanning confocal microscope (Carl Zeiss). Confocal microscope settings for comparison of MATER expression between *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were identical. For Nile red staining, oocytes were quickly washed 5 times in M2 medium, 3 times in MEM alpha medium (Invitrogen), and then incubated with Nile red (Sigma, 5μg/1ml) in MEM alpha medium for 5 minutes. Oocytes were briefly washed in PBS/PVA and PBS, attached to MatTek dishes (MatTek Corporation), which were then filled with MEM alpha. All procedures were carried out at room temperature.

Gel Filtration Chromatography

Eighty GV oocytes were collected from ovaries of CD-1 female mice. Using an AKTApurifier FPLC System (GE Healthcare), oocyte lysates were chromatographed on a superpose 6 K9/30 column pre-equilibrated with several column volumes of chromatography buffer containing 154mM NaCl, 50mM Na phosphate (PH 7.4), 5mM EDTA, and 0.02% NaN3. Elution positions of molecular weight standards (Sigma) were used to calibrate the column and determine the void volume. Fractions (1.0 ml) were transferred to a PVDF membrane by slot blot manifold (Hoefer PR 648) and analyzed by immunoblot. FPLC experiments were repeated two times. The immunoblot was scanned and relative intensity of bands was measured by ImageJ.

Western blotting

Mater$^{+/+}$ and *Mater*$^{tm/tm}$ GV oocytes (15 for non-extracted and 25 for extracted), were collected, lysed in 4X Laemmli buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.25M Tris HCl pH 6.8, 0.008% bromophenol blue), and boiled for 10 minutes at 100°C. Samples were run on a 10% SDS-PAGE gel at 100-120 V and transferred to PVDF membranes at 250mA for 2 hours. Membranes were blocked with 5% milk in 0.1 % TBS-T overnight at 4°C and subsequently incubated with PADI6 (1:13,000), MATER (1:12,000), and β-actin (1:1,000, Abcam) antibodies for 1-2 hour followed by anti-guinea pig (1:15,000), anti-rabbit (1:15,000) HRP antibody (Jackson ImmunoResearch) for 1 hour. Blots were developed using Immobilon Western HRP Chemiluminescent Substrate (Millipore). Films were scanned and densitometry was performed by Adobe photoshop CS4 program.

Transmission electron microscopy

Electron microscopy was performed as described elsewhere (24) with the following modifications. GV oocytes were collected from age-matched 4-6 week old *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ mice (three mice each) and immediately fixed with 2.5% Glutaraldehyde (EM Sciences), 4% PFA, 0.1% tannic acid and 0.01M $MgCl_2$ in 0.1M sodium cacodylate buffer (pH 7.3) at room temperature for 2 hours then overnight at 4°C. The oocytes were post-fixed with 1% osmium teroxide in cacodylate buffer for 1 hour, en bloc stained with 2% uranyl acetate, dehydrated in a graded ethanol series and then embedded in LX-112 resin (Ladd Research). Thin sections (50-70nm) were cut with a diamond knife (Diatome) on an AO/Reichert ultramicrotome and picked up on nickel 200 mesh thin bar grids. Grids are contrast stained with 2% uranyl acetate followed by Sato's modified lead stain. Samples were examined by a FEI T12 TWIN

transmission electron microscopy (TEM) at 100kV and images were collected with a Gatan Orius® dual-scan CCD camera.

Morphometric analysis

For morphometric analysis, TEM images (x 11,500) were overlayed with a 2106 cross point grid (0.8 cm spacing) using ImageJ, and points occupied by CPLs, LDs, mitochondria, and ER were scored using the point-counting method (29). The scoring was performed on 18 randomly spaced thin sections, with 3 sections from each of 6 different oocytes, which were obtained from 3 independent biological replicates of both *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ mice.

Statistical analysis

Means and standard error of the mean (SEM) were calculated for organelle volume fraction of *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes and statistical significance was calculated using a two-tailed *t*-test in Microsoft Excel 2007 program. P-value<0.05 was regarded as statistically significant. All experiments were repeated at least three times unless written otherwise.

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CHAPTER III

THE ROLE OF MATER IN ENDOPLASMIC RETICULUM DISTRIBUTION AND CALCIUM HOMEOSTASIS IN MOUSE OOCYTES

Abstract

Ca^{2+} oscillations are a hallmark of mammalian fertilization and play a central role in the activation of development. The calcium required for these oscillations is primarily derived from the endoplasmic reticulum (ER), which accumulates in clusters at the microvillar subcortex during oocyte maturation. The migration of the ER to the cortex during maturation is thought to play an important role in rendering the ER competent to generate the calcium transients and this redistribution of ER is thought to be primarily mediated by microtubules and microfilaments. We have previously shown that the oocyte- and early embryo-restricted maternal effect gene *Mater (Nlrp5)* localizes to, and is required for, formation of the oocyte cytoplasmic lattices, a tubulin containing structure that appears to play an important role in organelle positioning and distribution during oocyte maturation. Given these observations, we hypothesized that *Mater* may be also be required for ER redistribution and Ca^{2+} homeostasis in oocytes. To test this hypothesis, we first investigated ER localization in metaphase-II $Mater^{tm/tm}$ oocytes and found that, as opposed to wild type oocytes, ER clusters were not concentrated at the microvillar cortex. We next investigated if the pattern of Ca^{2+} oscillations was altered in $Mater^{tm/tm}$ oocytes after fertilization *in vitro*. Intriguingly, the Ca^{2+} oscillation patterns in $Mater^{tm/tm}$ oocytes showed a significantly lower amplitude of the first peak and higher frequency when compared to wild type oocytes. We then found that the Ca^{2+} oscillation defect in $Mater^{tm/tm}$ oocytes was likely caused by a reduced amount of intracellular Ca^{2+} stores in the ER using ionomycin and thapsigargin. Additionally, we found that, while the level of IP_3R-I (an essential mediator of ER-derived Ca^{2+}) in $Mater^{tm/tm}$ oocytes was similar to that of $Mater^{+/+}$ oocytes, the localization of IP_3R-I was altered in mutant eggs. To investigate the potential mechanisms by which MATER mediates ER redistribution, we also tested whether tubulin levels/localization was affected in the

mutant oocytes and found that Triton-insoluble fraction of Tubulin was significantly decreased in *Mater*$^{tm/tm}$ oocytes. Taken together, these observations support the hypothesis that MATER is required for ER distribution and Ca^{2+} homeostasis in oocytes, likely due to defects in lattice-mediated ER positioning and/or redistribution.

Introduction

Ca^{2+} oscillations are a hallmark of mammalian fertilization and initiate the transition of oocytes into early embryos (1-3). Calcium release is initiated when sperm release phospholipase C zeta (PLCζ) into the oocyte cytoplasm (4). PLCζ then cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) into 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). Binding of IP_3 to IP_3 receptors (IP_3Rs) located at the ER membranes elicits the release of Ca^{2+} from the ER resulting in a rise of intracellular Ca^{2+} ($[Ca^{2+}_i]$ (5). This initial rise in $[Ca^{2+}_i]$ is followed by repetitive $[Ca^{2+}_i]$ transients, termed Ca^{2+}oscillations, which stimulate egg activation and embryonic development (6,7). While the role of $[Ca^{2+}_i]$ transients in the early stages of the activation of development are now fairly well described in mammals, the role of Ca^{2+} oscillation patterns in later stages of embryonic development are now only beginning to be understood (8).

Metaphase II (MII) arrested oocytes show a higher level of IP_3-induced Ca^{2+} release at fertilization compared to GV stage oocytes (9,10). This finding indicates that changes occur during oocyte maturation to promote optimized IP_3- induced Ca^{2+} transients. These changes include; upregulation and post-translational modification of IP_3Rs, an increase of Ca^{2+} stores, and a redistribution of the endoplasmic reticulum (ER) to the microvillar cortex. More specifically, Ca^{2+} stores in the ER have been shown to increase during maturation and these

increases are directly correlated with optimized Ca^{2+} oscillation patterns at fertilization (11,12). Additionally, expression levels and phosphorylation of IP_3R-1, the primary IP_3R isoform in mammalian oocytes, increases during maturation and these increases are related to IP_3R-1 sensitivity (13-15). Such molecular changes are accompanied by a redistribution of the ER from a more evenly distributed pattern at the GV stage to more polarized pattern that is concentrated at the vegetal cortex in metaphase II-arrested oocytes (16,17). Given that the initial fertilization-induced Ca^{2+} rise is seen to propagate from this region after sperm-egg fusion, it is generally believed that these cortical ER clusters are the primary source of calcium for the oscillation. Importantly, the redistrubtion of ER during oocyte maturation is conserved across wide ranges of species, suggesting that it is likely fundamental functional calcium oscillations (16,18-22).

Regarding the mechanisms by which the ER redistributes during oocyte maturation, previous studies have shown that microtubules (MTs) appear to be essential for this process (16,23,24). MTs, along with microfilaments, mediate two major redistributions of the ER during oocyte maturation from a dispersed ER pattern at the GV stage, to a congression of ER around the nucleus during germinal vesicle breakdown (GVBD), and finally to an accumulation of ~1-2μm clusters of ER appearing at the cortex of mature MII-arrested oocytes. However, studies investigating the role of the cytoskeleton in ER redistributing remain limited. Given that the dynamic redistribution of organelles during oocyte maturation occurs within an exceedingly large volume (relative to somatic cells), it stands to reason that the oocyte may have evolved a specific structure to help facilitate organelle positioning and distribution. Recent studies suggest that the oocyte cytoplasmic lattices may represent just such a structure. The cytoplasmic lattices first appear as oocytes start to grow and eventually become a dominant feature of the mature

metaphase II-arrested mouse oocyte (25-27). More recently, the maternal effect gene, peptidylarginine deiminase 6 (PADI6), was found to localize to the lattices, thus providing insight into the molecular nature of this structure (28). PADI6 is an oocyte and early embryo abundant maternal effect gene that is required for female fertility (29). Through use of *Padi6* knockout mice, we have found that PADI6 is required for lattice formation and that the lattices appear to contain or regulate a stable form of non-spindle associated microtubules (30). Further, we also found that targeting of the ER and mitochondria to the oocyte cortex and the peri-spindle regions during maturation was defective in *Padi6* mutant oocytes, thus suggesting that PADI6 and the lattices play an important role in microtubule-mediated organelle redistribution.

MATER represents another oocyte and embryo abundant maternal effect gene that is essential for female fertility (31). We recently showed that, similar to PADI6, MATER also localizes to the oocyte lattices and is required for lattice formation (32). Given our findings with PADI6, here we decided to test whether similar organelle redistribution defects may occur in mutant *Mater* oocytes. Additionally, given the requirement of cortical ER clustering for optimal calcium signaling in mature oocytes, we also tested whether Ca^{2+} homeostasis was defective in mutant *Mater* oocytes. Outcomes from our study indicate that both ER positioning and Ca^{2+} homeostasis do appear to be significantly altered in mutant *Mater* oocytes. These findings provide new insight into the molecular mechanisms driving organelle positioning and function in the mammalian oocyte.

Results

Our previous findings with PADI6 (30) suggested that organelle positioning defects in *Mater*$^{tm/tm}$ oocytes might only become manifest after the onset of oocyte maturation. To test this hypothesis, we first stained fully-grown GV stage *Mater*$^{tm/tm}$ oocytes with ER tracker and imaged the oocytes by confocal fluorescence microscopy. Results showed that the ER staining patterns appeared similar between *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes (Fig. 1A). This result suggested that, similar to PADI6, ER positioning in immature GV stage oocytes does not require MATER. We then investigated ER distribution in mature *Mater*$^{tm/tm}$ MII oocytes using ER tracker. Here, we found that, as opposed to GV stage oocytes, ER distribution appeared to be affected by depletion of *Mater*. More specifically, we observed that, while the ER was concentrated in cortical clusters at the oocyte microvillar region in wild type oocytes, a non-polarized ER distribution pattern was observed in mature *Mater*$^{tm/tm}$ MII oocytes, with a markedly reduced number of cortical ER clusters (Fig. 1A). To further test whether ER localization was affected in mutant *Mater* oocytes, we next microinjected MII oocytes with the dicarbocyanine dye DiI_{18} (DiI), a fluorescent lipophilic dye, and imaged DiI-injected oocytes using confocal microscopy (33). We found that the DiI staining in *Mater*$^{+/+}$ oocytes was more polarized with cortical clusters of ER being seen at the oocyte cortex, which is in line with previous reports (16,33,34). However, in *Mater*$^{tm/tm}$ MII oocytes, the ER was distributed in a more non-polarized manner with the number of cortical ER clusters being reduced (Fig. 1B). In order to gain insight into the dynamics of ER movements in mature *Mater*$^{tm/tm}$ oocytes, we then imaged the DiI-stained oocytes by time-lapse microscopy at 10 second intervals for 20 minutes. Results show that that the ER cortical clusters appear to be stably localized to the cortex of wild-type oocytes (Fig. 2). However, in the mutant oocytes, ER clusters are seen throughout the interior of the oocyte cytoplasm, but they do not appear to be targeted to the oocyte cortex.

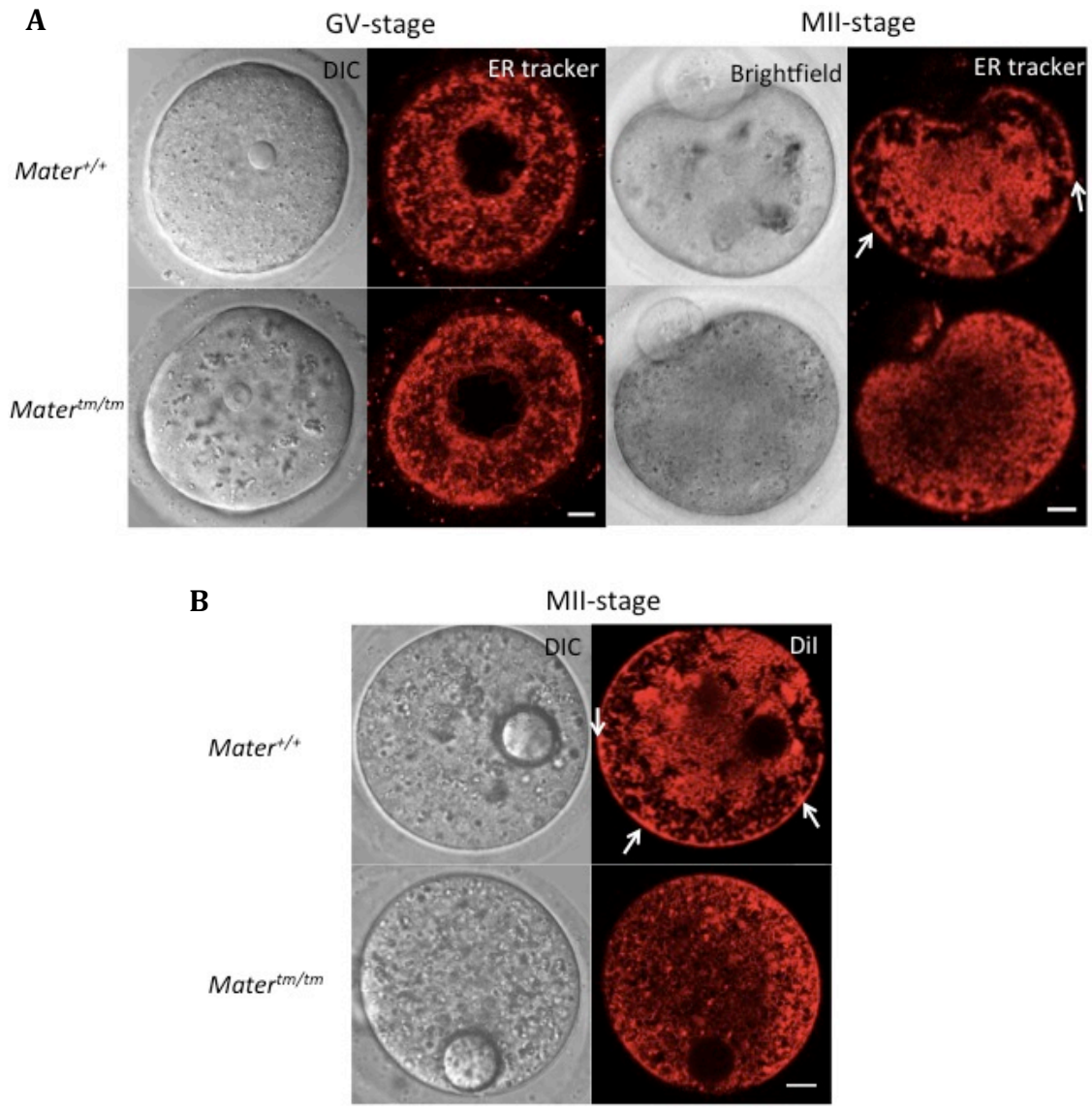


Figure 3.1. Altered endoplasmic reticulum localization and decreased cortical ER clustering in metaphase II-arrested *Mater*$^{tm/tm}$ oocytes

(A) GV stage and MII stage *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were stained with ER tracker and imaged using confocal microscopy. (**B)** DiI was microinjected into mature MII-arrested *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes and the localization of DiI (an ER marker) was documented using confocal microscopy. DIC images of microinjected oocytes highlight the DiI-containing oil-drop. Arrows highlight the cortical ER clusters in *Mater*$^{+/+}$ oocytes, which are markedly reduced in *Mater*$^{tm/tm}$ oocytes. Scale bar 10μm

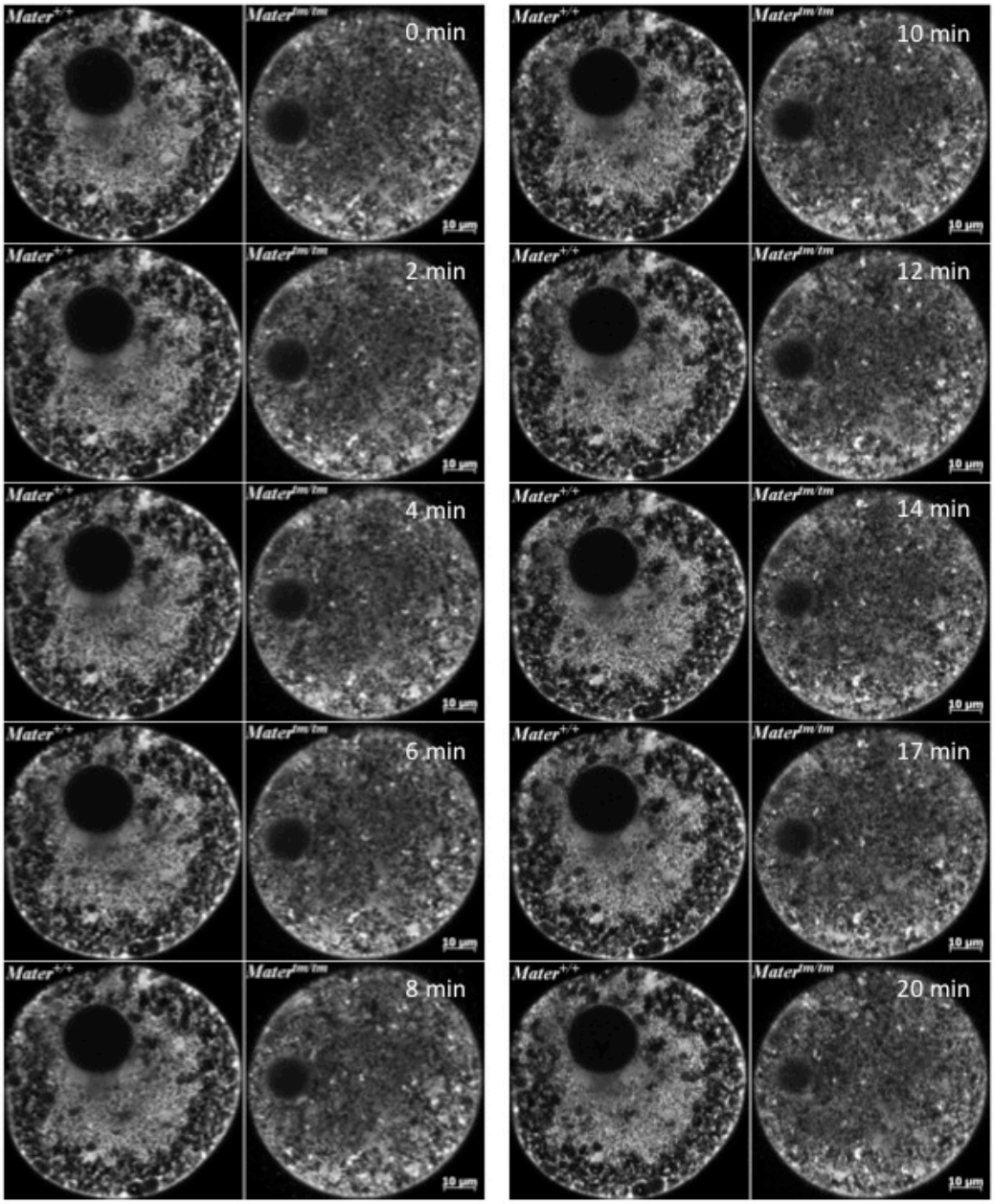


Figure 3.2. Endoplasmic reticulum dynamics in mature metaphase II-arrested *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes.

MII oocytes were microinjected with the ER marker, DiI, washed in HEPES-CZB, attached to Mattek dishes, and subjected to time-lapse imaging. Images (63x objective) were obtained every 10 sec for 20 minutes using a confocal microscope. Images at specific time points are shown.

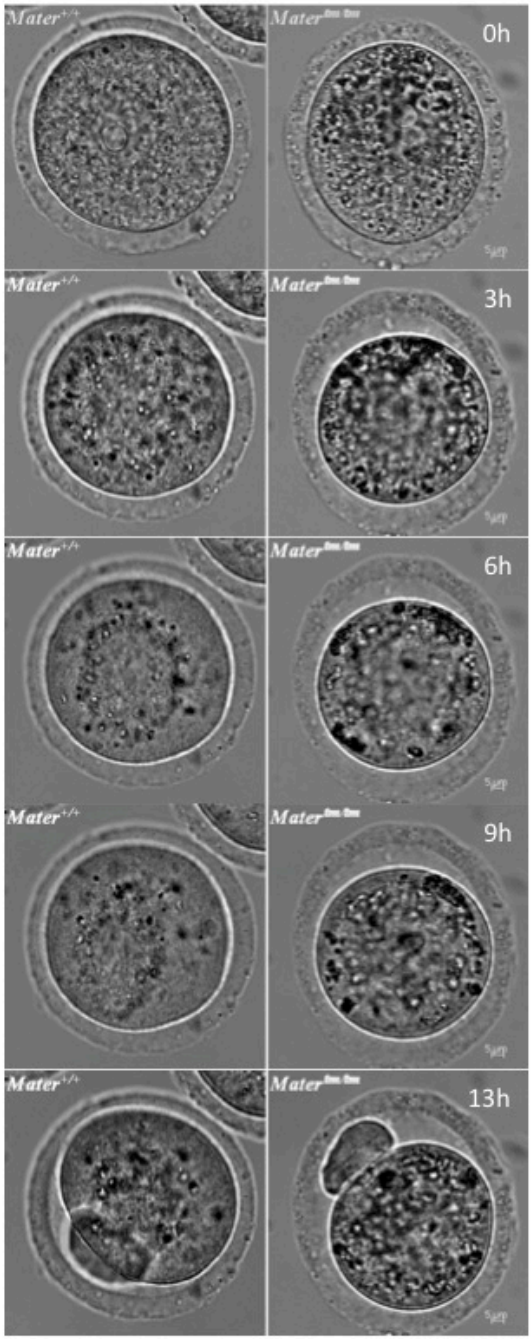


Figure 3.3. Dynamics of lipid droplets during maturation *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes *in vitro.*

GV-stage *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were matured in M16 media at 37°C and 5% CO_2. Time-lapse images were obtained every 1min for 13h using wide-field microscopy and images at specific time points are shown.

Previous studies have shown that microtubules are major drivers of ER redistribution during oocyte maturation. We showed previously that PADI6 interacts with tubulin at the oocyte lattices and that PADI6 and the lattices appear to play a critical role in ER redistribution during oocyte maturation (34). These findings suggest that the lattices play an important role in microtubule-mediated organelle redistribution during maturation. Therefore, to gain more insight into the potential mechanisms behind the observed organelle redistribution failure in *Mater*$^{tm/tm}$ oocytes, we tested whether the localization and/or levels of tubulin were altered in *Mater*$^{tm/tm}$ oocytes. We first investigated whether MATER co-localized with tubulin in Triton X-100 extracted *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes. GV oocytes were extracted for 10 minutes with 0.1% Triton X-100, fixed, and stained with anti-tubulin and anti-MATER antibodies. Confocal imaging of the oocytes showed that, similar to PADI6, MATER co-localized with non-MT fraction of tubulin in the extracted GV oocytes (Fig. 4A), and that, while the total amount of tubulin was not affected by depletion of MATER, the amount of Triton-insoluble tubulin was markedly reduced in *Mater*$^{tm/tm}$ GV stage oocytes (Fig. 4C, 4D). These results support the hypothesis that MATER plays an important role microtubule dynamics in oocytes.

Targeting of the ER to subcortical clusters during oocyte maturation is thought to play a major role in ER-mediated Ca^{2+} signaling at fertilization. The finding that mature MII-arrested *Mater*$^{tm/tm}$ oocytes contain a more diffuse ER distribution pattern lead to the hypothesis that ER function, particularly as a Ca^{2+} source, might be compromised in *Mater*$^{tm/tm}$ oocytes. Therefore, we measured intracellular Ca^{2+} stores in GV and MII oocytes to determine if Ca^{2+} storage was affected in *Mater*$^{tm/tm}$ oocytes at either of these stages. Oocytes were loaded with the Ca^{2+} binding dye, Fluo-4AM (Fluo-4) and treated with ionomycin (1μM) to release intracellular Ca^{2+}

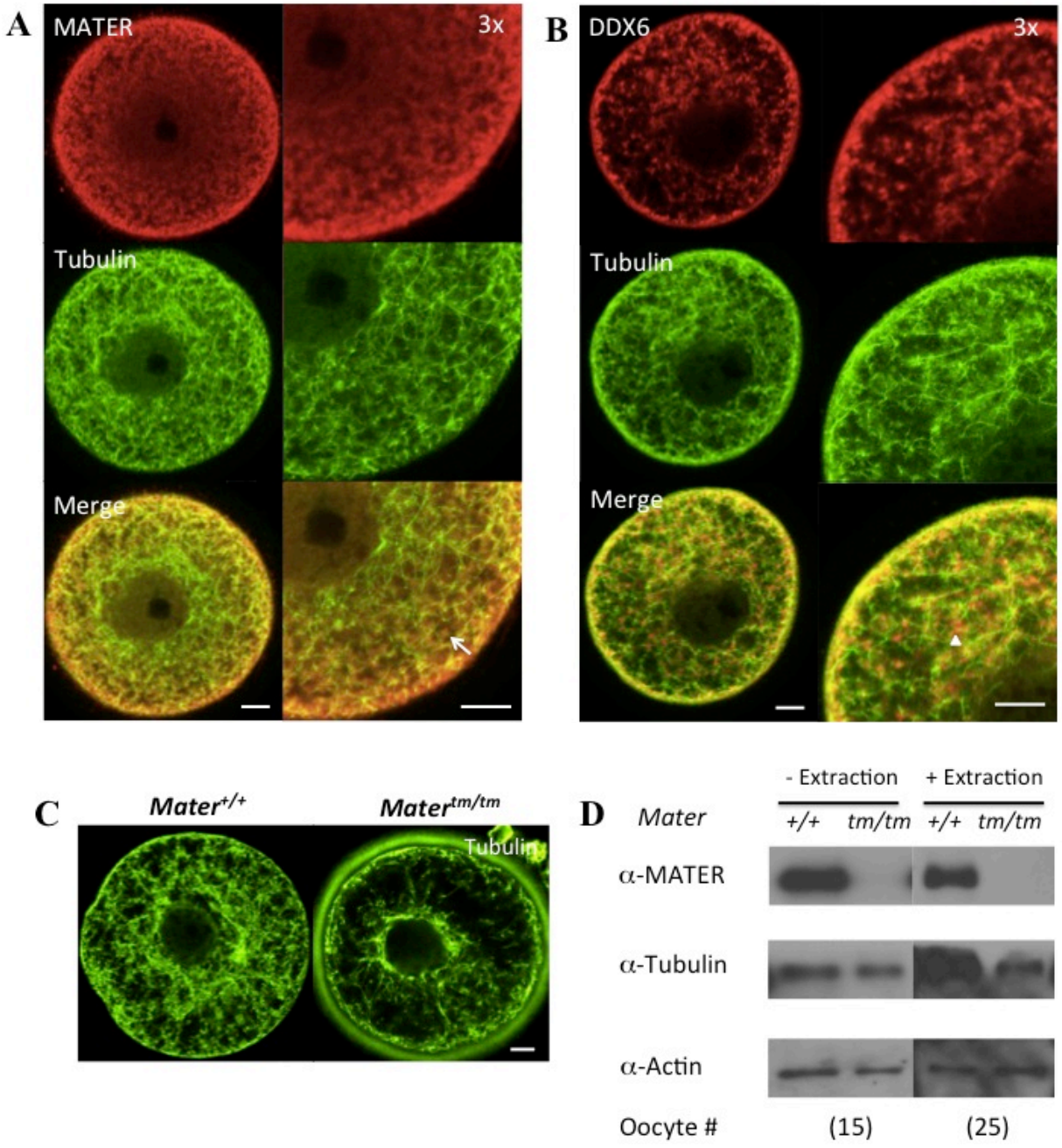


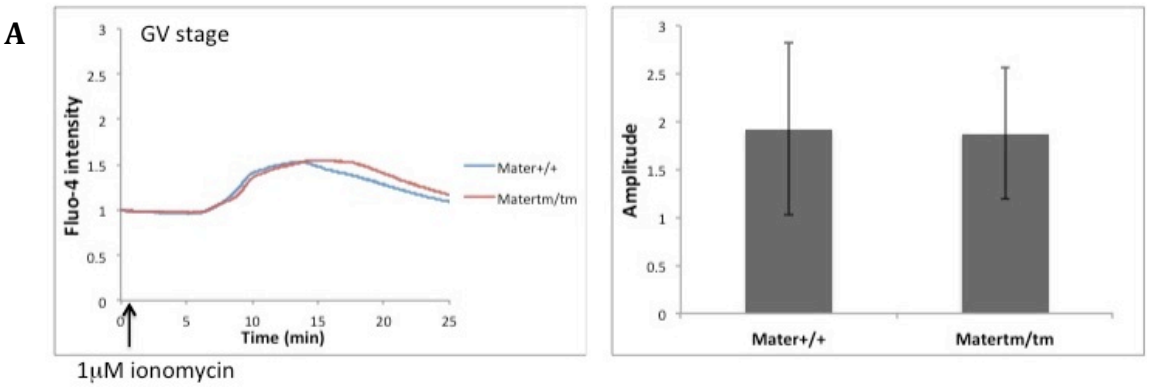
Fig 3.4. MATER and tubulin appear to be closely associated with each other in oocytes

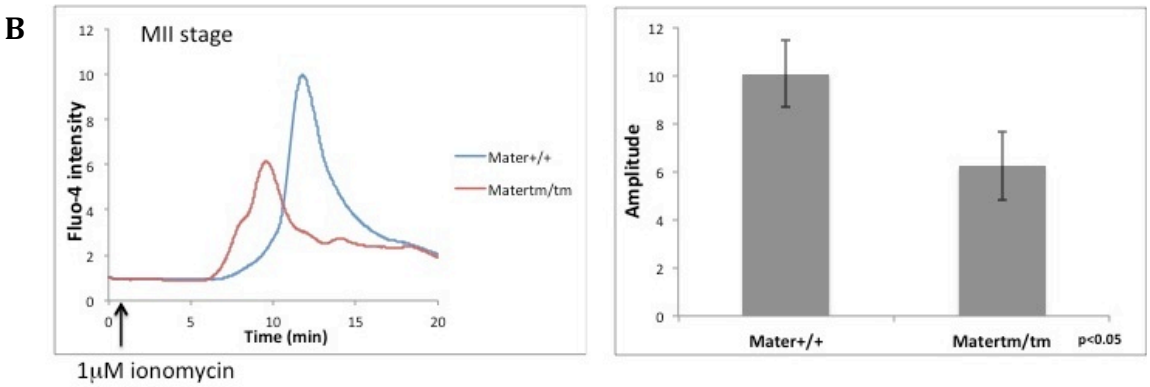
(**A)** Confocal microscopy imaging of Triton X-100 extracted GV-stage oocytes stained with anti-MATER (red) and anti-alpha-tubulin antibodies (green). Merge images show that MATER appears to co-localizate with tubulin in the non-microtubule (MT) associated fraction of Triton extracted oocytes. Tubulin and MATER co-localization is highlighted by the arrow in the 3x

zoom image at left. **(B)** The co-localization between tubulin and DDX6 (red, used here as a control) is shown in the right panels. The arrowhead in the 3x zoom image highlights the lack of co-localization between DDX6 and tubulin. Scale bar = 10μm. **(C)** Confocal microscopy images of *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes following Triton X-100 extraction. The amount of Triton-insoluble tubulin (green) is decreased in *Mater*$^{tm/tm}$ oocytes compared to that of *Mater*$^{+/+}$. **(D)** Western blotting indicates that the Triton-insoluble fraction of tubulin is sharply reduced in *Mater*$^{tm/tm}$ oocytes compared to wild type oocytes. Total tubulin levels, however do not appear to be different between mutant and control oocytes.

stores. The calcium release was then captured by confocal microscopy using a 488nm argon laser and BP 505-550nm filters. The traces of Fluo-4 were then normalized to the background levels, and analyzed by imageJ. Results show that GV-stage *Mater*$^{tm/tm}$ oocytes responded to ionomycin to a similar degree as *Mater*$^{+/+}$ oocytes (Fig. 5A). However, we found that, while treatment of wild-type metaphase II-arrested oocytes with ionomycin lead to an ~10-fold increase of the Fluo4 cytoplasmic intensity, Fluo4 staining in hypomorph oocytes was only increased by ~ 6-fold, indicating that depletion of MATER lead to a ~1.7 fold decrease in intracellular Ca^{2+} stores (Fig. 5B). To investigate ER Ca^{2+} stores in hypomorph oocytes, we treated MII oocytes with 5μM thapsigargin, which specifically blocks the ER Ca^{2+}-ATPase pump (SERCA) (35). Similar to ionomycin treatment, we found that *Mater*$^{tm/tm}$ MII oocytes demonstrated a 30% decrease in cytoplasmic calcium in response to thapsigargin treatment compared to wild type oocytes (Fig. 5C). These results suggest that the observed organelle redistribution defect in *Mater* hypomorph oocytes might correlate with the inability of the ER to store calcium during oocyte maturation.

Several lines of evidence indicate that the IP_3R-I is a major receptor for releasing Ca^{2+} from the ER in mammalian oocytes (14,36). Given our finding the ER distribution is defective in mutant oocytes and that IP_3R-I is primarily thought to localize to the ER, we next investigated the localization of IP_3R-I in *Mater*$^{+/+}$ and $^{tm/tm}$ MII oocytes by confocal microscopy. Oocytes were fixed, and stained with IP_3R-I specific antibody. Results show that the localization of IP_3R-I is diffuse throughout the cytoplasm in *Mater*$^{tm/tm}$ oocytes with significantly reduced levels of IP_3R-I clusters at the oocyte cortex (Fig. 6A). We then sought to determine the abundance of IP_3R-I protein in *Mater*$^{+/+}$ and $^{tm/tm}$ MII oocytes by immunoblotting. Oocytes were collected, lysed, and were subjected for immunoblotting. The blots were then incubated with the anti-Rbt03 antibody


A
GV stage
Fluo-4 intensity
Time (min)
Mater+/+
Matertm/tm
Amplitude
Mater+/+
Matertm/tm
1μM ionomycin


B
MII stage
Fluo-4 intensity
Time (min)
Mater+/+
Matertm/tm
Amplitude
Mater+/+
Matertm/tm
p<0.05
1μM ionomycin

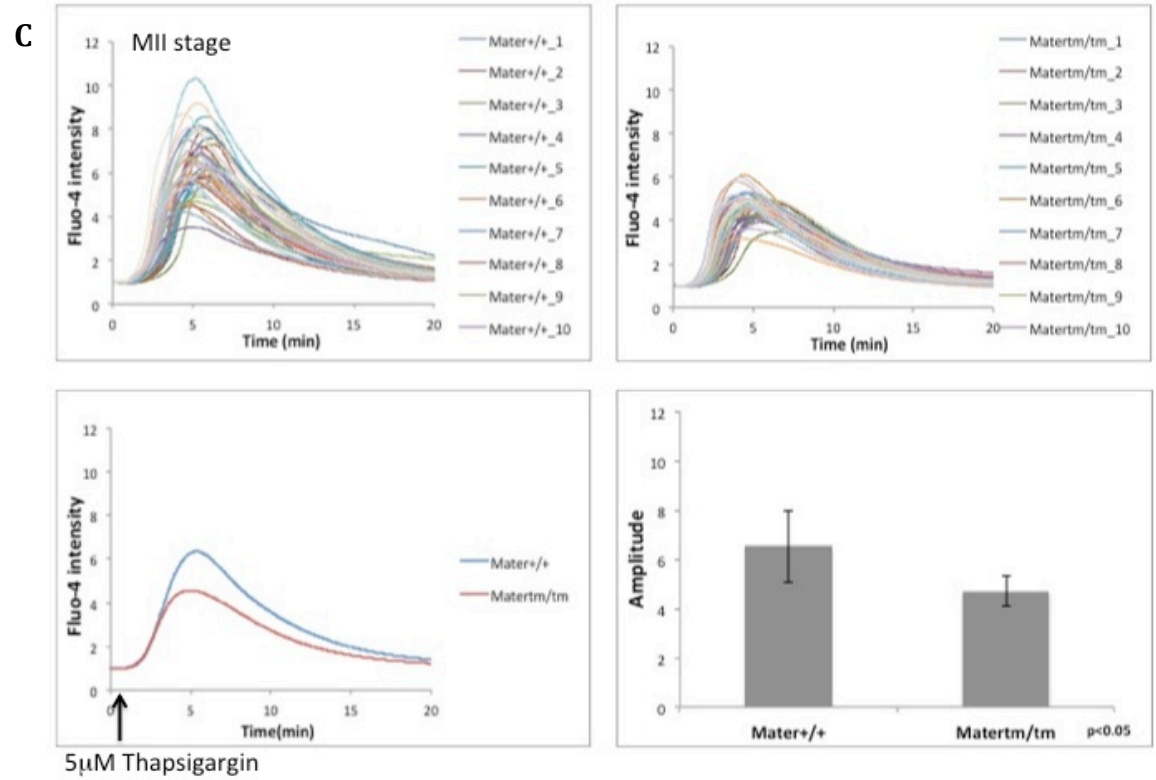

C
MII stage
Fluo-4 intensity
Time (min)
Mater+/+_1
Mater+/+_2
Mater+/+_3
Mater+/+_4
Mater+/+_5
Mater+/+_6
Mater+/+_7
Mater+/+_8
Mater+/+_9
Mater+/+_10
Matertm/tm_1
Matertm/tm_2
Matertm/tm_3
Matertm/tm_4
Matertm/tm_5
Matertm/tm_6
Matertm/tm_7
Matertm/tm_8
Matertm/tm_9
Matertm/tm_10
Mater+/+
Matertm/tm
Time(min)
Amplitude
Mater+/+
Matertm/tm
p<0.05
5μM Thapsigargin

Figure 3.5. Intracellular Ca^{2+} stores are decreased in *Mater*$^{tm/tm}$ oocytes

(A) GV oocytes were loaded with Fluo-4 AM (1μM), treated with ionomycin (arrow indicates time of treatment) and imaged at ~1.6 second intervals using 20x objective. Histogram of Fluo-4 intensity shows that GV-stage *Mater*$^{tm/tm}$ oocytes responded to ionomycin to a similar degree as *Mater*$^{+/+}$ oocytes. (**B)** MII-arrested *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were treated with ionomycin (1μM) as above. Results show that *Mater*$^{tm/tm}$ oocytes responded to ionomycin to a significantly lesser degree compared to *Mater*$^{+/+}$ oocytes. $p<0.05$ (**C)** MII *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were treated with thapsigargin (5μM) as above. Traces of $[Ca^{2+}]_i$ changes in individual *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes are shown at top. Average of $[Ca^{2+}]_i$ changes are shown below. Similar to ionomycin treatment, the responses of *Mater*$^{tm/tm}$ oocytes to thapsigargin were markedly decreased when compared to *Mater*$^{+/+}$ oocytes ($p<0.05$).

to detect IP_3R-I, and an anti-actin antibody was employed as a loading control. Result indicates that expression level of IP_3R-I in *Mater*$^{tm/tm}$ oocytes seems to be equivalent to that of *Mater*$^{+/+}$ oocytes (Fig. 6B). These findings suggest that, while IP_3R-I protein levels are not affected by MATER depletion, the localization of IP_3R-I does appear to be significantly altered in these oocytes, thus providing further mechanistic insight into the observed Ca^{2+} homeostasis defect in mutant oocytes.

Ca^{2+} oscillations are dependent on intracellular Ca^{2+} stores and are believed to have a spatio-temporal relationship with clustering of these stores, mainly ER, at oocyte cortex. Given the observed decrease in ER cortical clusters and intracellular Ca^{2+} stores in *Mater*$^{tm/tm}$ oocytes, we next tested whether Ca^{2+} oscillation patterns might be altered in *Mater* hypomorph oocytes following fertilization. MII oocytes were treated with acid tyrodes to remove the zona pellucida and then loaded with Fluo-4. Next, oocytes were inseminated (~100 sperm per μl) with capacitated epididymal sperm and the Fluo-4 signal was captured at 20 second intervals for 1 hour. *In vitro* fertilization was confirmed by the visualization of the extruded polar body ~2h following fertilization. The Ca^{2+} oscillation profiles of *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were then analyzed using ImageJ and representative Ca^{2+} oscillations from each group are shown (Fig. 7A). Then the amplitude of the first peak was averaged and the frequency of Ca^{2+} oscillations were calculated. Results showed that the initial Fluo-4 peak in *Mater*$^{tm/tm}$ oocytes was reduced by ~46% compared to *Mater*$^{+/+}$ oocytes. Additionally, we found that the frequency of subsequent Ca^{2+} oscillations was about 50% higher in *Mater*$^{tm/tm}$ oocytes compared to *Mater*$^{+/+}$ oocytes (Fig. 7B). These results suggest that depletion of *Mater* significantly hampered the oocytes ability to

generate normal Ca^{2+} oscillations, with hypomorph oocytes showing a Ca^{2+} oscillation profile that was of lower amplitude and higher frequency.

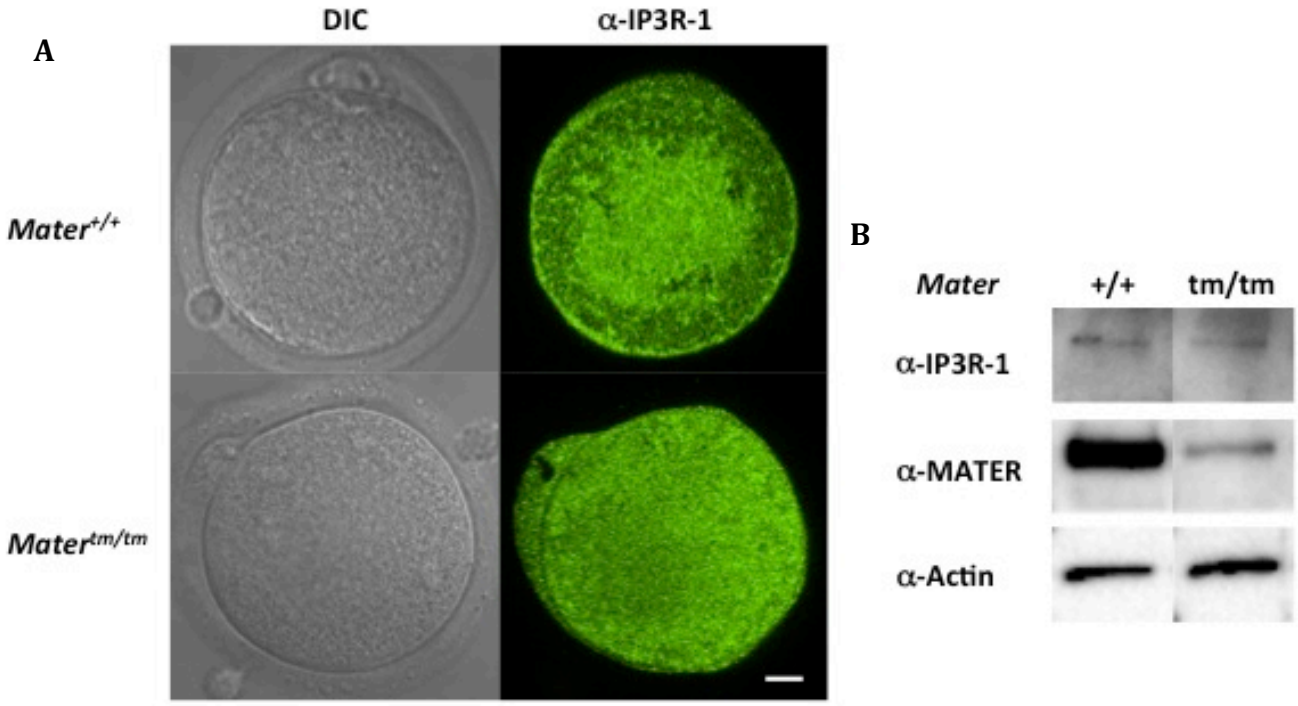


Fig 3.6. IP_3R-I distribution is altered in *Mater*$^{tm/tm}$ oocytes

(A) MII-arrested *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100, and stained with an anti-IP_3R-I antibody. Results show that the localization of IP_3R-I is altered in the *Mater*$^{tm/tm}$ oocytes when compared to wild type oocytes. Scale bar: 10μm **(B)** Proteins from *Mater*$^{+/+}$and *Mater*$^{tm/tm}$ oocytes were resolved by SDS-PAGE, and transferred to PVDF membrane. The membrane was probed with anti-IP3R-I, MATER, and actin antibodies. Results show that IP_3R-I levels do not appear to be significantly reduced in *Mater*$^{tm/tm}$ oocytes when compared to wild type oocytes.

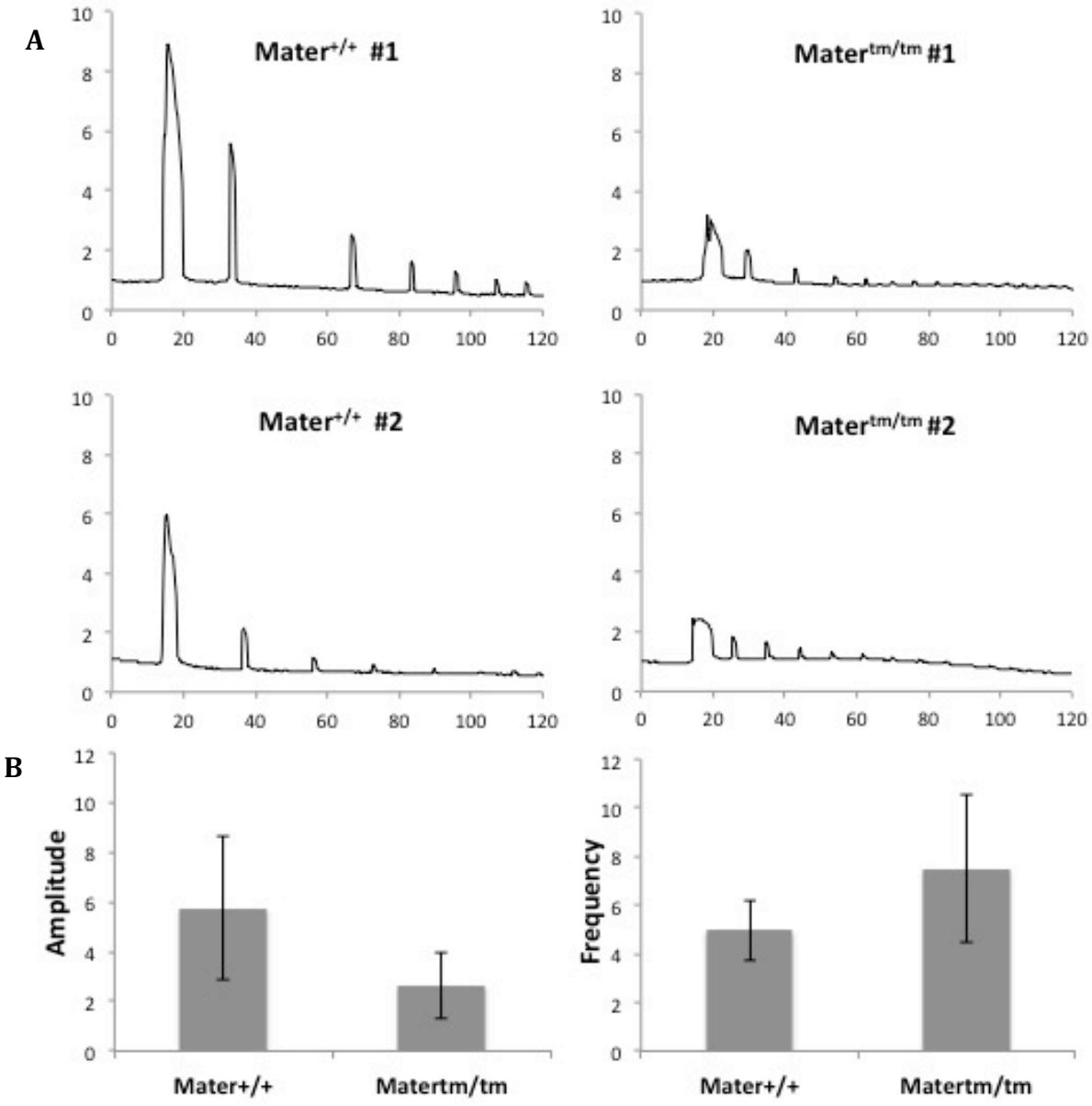


Figure 3.7. ***Mater*$^{tm/tm}$ oocytes show an altered Ca^{2+} oscillation pattern following fertilization *in vitro.***

Zona-free metaphase II-arrested *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes were loaded with Fluo-4 AM, fertilized *in vitro* and Ca^{2+} oscillations were recorded every 20 second for one hour following insemination. Fertilization was confirmed by the presence of second polar body extrusion 2h

after insemination. Images were analysed by ImageJ and the representative graphs from each group are shown in **(A)**. The bar graphs of amplitude and frequency in **(B)** indicate that *Mater*$^{tm/tm}$ oocytes and display a lower amplitude and higher frequency of Ca^{2+} oscillation patterns when compared to *Mater*$^{+/+}$ oocytes ($p<0.05$).

Discussion

Cytoplasmic lattices (CPLs) are mammalian oocyte- and embryo- abundant structures, which consist of 5-7 ~25nm parallel fibers with each fiber ranging from 200-1000 nm in length (37). We recently documented that PADI6 localizes to the lattices and is required for lattice formation, thus providing insight into the molecular composition of this enigmatic structure. Additionally, we have also shown that tubulin interacts with PADI6 and co-localizes with PADI6 at the CPLs. Further, we also found that the Triton-insoluble fraction of tubulin is significantly reduced in *Padi6*$^{-/-}$ oocytes, thus strengthening the argument that tubulin is also a major component of the lattices (34). We have also recently reported that another maternal effect gene, MATER, co-localizes with PADI6 and is required for lattice formation (38). In this report, we expand upon these findings by showing that, similar to PADI6, MATER also co-localizes with the non-MT fraction of tubulin in extracted GV oocytes, and that Triton insoluble fraction of tubulin is markedly reduced in *Mater*$^{tm/tm}$ GV oocytes. These findings further strengthen the premise that tubulin is a component of lattices. While the precise relationship between microtubules and the lattices remains to be resolved, our findings to date suggest that the lattices likely play a direct role in microtubule dynamics.

Interestingly, another set of studies has identified MATER as a member of the Subcortical maternal complex (SCMC), which also contains FLOPED, TLE6, and FILIA. PADI6 was also identified in these studies as a putative SCMC member (39). Recently, FLOPED was found by another research group to localize to the lattices and be required for lattice formation (40). The observation that PADI6, MATER, and FLOPED are putative components of the SCMC and the lattices strongly suggests that these two entities are connected in some way. We have found that

the localization of MATER and PADI6 can vary dramatically depending on the preparation/fixation methods utilized and that, under more stringent conditions, MATER and PADI6 do not appear to be cortically localized and, instead are more evenly distributed throughout the cytoplasm (41). Therefore, we predict that the SCMC likely represents a component of the lattice superstructure and may function to regulate tubulin dynamics. Interestingly, we previously found that levels of acetylated tubulin, a marker for the stable microtubules (MT) were significantly decreased in *Padi6*$^{-/-}$ oocytes, suggesting that the lattices might represent the stable or modified form of microtubules. In somatic cells, the ER has been shown to move throughout the cytoplasm along tracks of acetylated microtubules (42). Therefore, while speculative, it is possible that ER targeting to the cortex during maturation is carried out by the direct trafficking of the ER along the lattice superstructure itself.

During oocyte maturation the Ca^{2+} releasing machinery goes through its own maturation process in order to mount an optimal Ca^{2+} response at fertilization with much of this maturation process occurring at the level of the ER (43). In addition to events such as IP_3R-I phosphorylation, cortical targeting and clustering of the ER is thought to be a key aspect of preparing the ER for an optimal Ca^{2+} response. This prediction is highlighted by the fact that ionomycin and, more specifically, thapsigargin treatment induces a much more robust cytosolic Ca^{2+} peak in mature MII-arrested oocytes compared to immature GV-stage oocytes (11). In this study, we found that calcium release in ionomycin and thapsigargin treated *Mater*$^{tm/tm}$ GV stage oocytes was similar to that of wild type oocytes. However, we found that calcium release was reduced by ~40% in mature MII-arrested *Mater*$^{tm/tm}$ oocytes compared to wild type oocytes. These findings support the hypothesis that the ability of the ER in *Mater*$^{tm/tm}$ oocytes to sequester and store calcium

during oocyte maturation is compromised, likely due to the inappropriate targeting of the ER to the cortex. Alternatively, however, given that thapsigargin inhibits SERCA, which is responsible for returning Ca^{2+} into ER, it is possible that SERCA expression levels or activity is compromised in *Mater*$^{tm/tm}$ oocytes. The finding that ionomycin mounted a similar response to that of thapsigargin in *Mater*$^{tm/tm}$ oocytes, however, further supports the hypothesis that the weak calcium peak in hypomorph oocytes was actually due to the inability of these oocytes to store Ca^{2+}.

The finding that ionomycin and thapsigargin treatment of mature MII-arrested *Mater*$^{tm/tm}$ oocytes resulted in a ~2 fold decrease in cytosolic calcium suggested that fertilization-induced calcium oscillations would also be compromised in the mutant *Mater* oocytes. We tested this hypothesis and found that calcium oscillations were, in fact, sub-optimal; with a ~50% decrease in the initial calcium peak. Interestingly, in addition to this weak initial peak the frequency of subsequent oscillations was found to be increased in the *Mater* hypomorph oocytes. These findings raise the possibility that the relatively weak calcium oscillations observed in *Mater*$^{tm/tm}$ oocytes compromises oocyte activation and, thus, subsequent stages of embryonic development.

Regarding which stages might be compromised; we found that, following fertilization, extrusion of the second polar body occurred without a noticeable delay in *Mater*$^{tm/tm}$ oocytes indicating that exit from meiosis did not appear to be compromised in these oocytes. Previous reports have documented that fertilized *Mater*$^{tm/tm}$ oocytes formation apparently normal pronuclei and undergo the first mitotic cleavage division and then arrest at the two-cell stage (44). These observations suggest that the calcium signals generated by *Mater*$^{tm/tm}$ oocytes are sufficient to

allow progression through the initial stages of oocyte activation, i.e. the resumption and progression of meiosis, extrusion of the second polar body, and proucleus formation.

However, while less understood calcium signaling is also known to be important for molecular changes that occur following fertilization and also for the acquisition of developmental competence during preimplanation development. For example, protein expressions in 1- and 2-cell embryos rely largely on the translation of stored maternal mRNAs (45). Interestingly, mRNA recruitment in early embryos appears to be coupled with Ca^{2+} oscillations (8,46) suggesting that fertilization-induced calcium oscillations temporally regulate the production of molecular factors that orchestrate early development. Previous studies have shown that embryos derived from *Mater*$^{tm/tm}$ oocytes show a reduction in *de novo* expression proteins such as those found in the Transcription requiring complex (TRC), a major marker of embryonic genome activation, and also display altered protein expression profiles (47) (48). Therefore, it is tempting to speculate that, while the initial calcium peak(s) in *Mater*$^{tm/tm}$ oocytes were sufficient to allow the oocytes to exit from meiosis and form pronuclei, these sub-optimal peaks, coupled with the increased frequency of the subsequent peaks, lead to a dis-regulated pattern of protein synthesis and an eventual two-cell arrest.

Regarding the role of calcium signaling in developmental competence, previous studies have shown that alteration of Ca^2 oscillation parameters such as a number, amplitude, or frequency in parthenogenetically activated oocytes can result in decreased rates of preimplantation development (49,50). Additionally, aged mouse oocytes, which show a reduced rate of blastocyst formation following IVF (51), were found to display altered Ca^{2+} oscillation patterns

with lower amplitude and higher frequency, and also contain reduced Ca^{2+} stores. This pattern appears remarkably similar to what we have observed for *Mater*$^{tm/tm}$ oocytes, thus raising the possibility that depletion of *Mater* may promote premature oocyte aging. In support of this hypothesis, decreased levels of MATER protein have been documented in aged oocytes(52).

While our report has focused on the role of MATER in ER positioning and distribution, another group has recently investigated the role of MATER in mitochondrial positioning and function. This study found that, similar to the ER defect described in our report, *Mater*$^{tm/tm}$ metaphase II-arrested oocytes also display an altered mitochondrial distribution pattern(53). Also, this group showed that mitochondria in and 2-cell embryos were dysfunctional. Given the well documented interplay between mitochondria and ER with respect to Ca^{2+} homeostasis, it is possible that the Ca^{2+} store defect described in our study could be attributed, in part, to the loss of a functional interaction between the ER and mitochondria in mutant oocytes. Importantly, this study also found that *Mater*$^{tm/tm}$ oocytes/zygotes appear to be under mitochondrial stress as documented by the upregulation of the phosphorylation in oxidative stress marker, p66Shc (54). Therefore, in this study we assessed whether *Mater*$^{tm/tm}$ oocytes are under ER stress by measuring protein levels of a canonical marker for ER stress, GRP78 (HSPA5), (55)(Fig.8). Results show that there is no discernible effect on GRP78 expression level between *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ MII oocytes, implying the defects of Ca^{2+} homeostasis shown in *Mater*$^{tm/tm}$ MII oocytes are not likely due to ER stress.

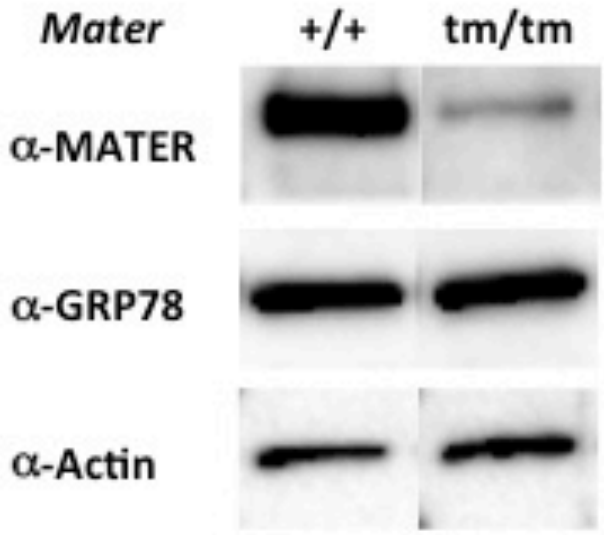


Figure 3.8. Protein level of GRP78 appears to be similar between *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ MII oocytes

MII-stage *Mater*$^{+/+}$ and *Mater*$^{tm/tm}$ oocytes (55) were lysed, separated by SDS-PAGE gel, and transferred to PVDF membranes. Blots were then incubated with anti-MATER, GRP78, and beta actin antibodies.The level of beta actin is used as a loading control.

In conclusion, our previous study found that MATER is required for formation of the tubulin-containing cytoplasmic lattices. Here we show that MATER colocalizes with tubulin in the Triton-insoluble cell fraction and that depletion of *Mater* results in increased tubulin solubility. These findings suggest that MATER plays an important role in tubulin dynamics. Additionally, we show that MATER is also required for the proper targeting and clustering of the ER at the microvillar cortex. While the mechanism behind this defect is currently unclear, the identified link between MATER and tubulin suggests that the ER positioning defect in mutant oocytes is due to an altered cytoskeleton. Additionally, we also document that MATER appears to be required for the proper storage of Ca^{2+} within the ER in mature MII-arrested oocytes. We attribute this defect to the inability of mutant oocytes to redistribute the ER during oocyte maturation. We also show that the pattern of fertilization-induced Ca^{2+} oscillations is defective in *Mater*$^{tm/tm}$ MII oocytes; with the mutant eggs showing a suppressed initial calcium peak followed by an increased frequency of subsequent peaks. We predict that the observed Ca^{2+}oscillation defects in mutant *Mater* oocytes are most likely due to the depleted Ca^{2+} stores in the ER. Lastly, we hypothesize that the altered Ca^{2+} oscillation pattern observed in fertilized *Mater*$^{tm/tm}$ oocytes likely plays an important role in the subsequent 2-cell developmental arrests and future experiments will directly test this hypothesis.

Materials & Methods

Mice

Oocytes were collected from *Mater*$^{+/+}$ and *Mater* $^{tm/tm}$ mice. The generation of MATER transgenic mice has been described elsewhere (44). CD-1 male mice were purchased from commercial vendors. Mouse colonies were housed in the ECRF mouse facility at Cornell University's College of Veterinary Medicine in accordance with Cornell animal care guidelines.

Collection of gametes

Oocytes. Germinal vesicle stage oocytes were collected from 4-6 week female mice in M2 media (supplemented with 200μM IBMX) approximately 46-48h after injection of 2.5-5IU pregnant mare serum gonadotrophin (PMSG). Metaphase II oocytes were collected 12.5-14h after injection of ~5 IU of human chorionic gonadotrophin (hCG) and cumulus cells were removed using 0.1% Hyaluronidase. For zona-free oocytes, MII eggs were collected in Tyrode-HEPES buffer with PVA. To remove the zona pellucida, eggs were briefly treated with acid tyrode solution (pH 1.6) and washed 3X in Tyrode-HEPES with PVA. **Sperm.** For *in vitro* fertilization, sperm were collected into 900μl of HTF media (supplemented with 4mg/ml BSA) from the caudal epididymis of retired CD1 breeding males. To capacitate sperm, 100μl of the sample was further diluted in 200μl HTF media and incubated for 2-3hours in 37°C incubator with 5% CO_2.

Ca^{2+} imaging

For Ca^{2+} imaging, oocytes were loaded with 1.5μM Fluo-4 AM (molecular probes) and 0.2% Pluronic F-127 in Tyrode-HEPES buffer with PVA for 20mins at room temperature. Oocytes were washed 3X with Ca^{2+} free Tyrode-HEPES (containing 1mM EGTA) and attached to poly-l-lysine coated Mattek dish for imaging. After baseline signals were recorded, thapsigargin (5μM) or ionomycin (1μM) was added to the media to detect cytosolic Ca^{2+} stores. Images were taken every 1.573second using an Argon laser-equipped confocal microscope. For imaging Ca^{2+} oscillations, eggs were attached to Mattek dish in 50μl of HTF media (without BSA) under mineral oil following brief washes in Tyrode-HEPES and HTF media. 50μl of HTF media (with 8mg/ml BSA) was then added to the dish and the eggs were inseminated with ~3000 sperm. Upon insemination, eggs were imaged for 1h every 20 sec using a Zeiss LSM 510 confocal microscope.

Lentiviral infection

Flag-tagged Mater was subcloned into a TetO-FUW vector and viral packaging plasmids psPAX2 and pMD2.G (plasmids were gifts from Dr. John Schimenti) were co-transfected with TetO-FUW-Mater, rtTA into 293T cells. Viral supernatants were collected 48h-72h after transfection, and rtTA and TetO-FUW-Mater were pooled together and concentrated. $3x10^5$ Cos-1 cells were seeded into 6 well plates one day prior to infection and cells were infected for 24h. 2μg/ml doxycycline was added into viral infected cells and assayed 48-72h later.

Western blotting

Western blotting procedure was described elsewhere (Kim et al. 2010). Briefly, oocytes were collected, lysed in Laemmli buffer, and boiled for 10 minutes at 100°C. For anti-Rbt03 western blotting, PVDF membranes were run in 7.5% PAGE gel, transferred overnight, blocked in TBST

with 5% milk for 2hr and incubated overnight with anti-Rbt03 antibody at 1:1000. Blots were developed using Immobilion Western HRP Chemiluminescent Substrate (Millipore) and Chemidoc MP (Biorad).

Microinjection

To label the endoplasmic reticulum, a saturated solution of DiI 18 (1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate 18) (Molecular Probes) was prepared in soybean oil (Sigma). The DiI solution was loaded into a Femtotips II injection pipette (Eppendorf) with a microloader (Eppendorf). Oocytes were pressure-injected with the micropipette and Eppendorf TransferMan NK2 manipulator mounted on an inverted microscope (Nikon). MII- oocytes were injected immediately after collection in M2. They were then placed in a drop of Hepes buffered CZB+IBMX covered with mineral oil during the inejction. A holding pipette (Humagen) was used to immobilize the oocytes and the injection pipette was pushed through the zona pellucida and plasma membrane. Each oocyte receives ~5pl of DiI solution via Eppendorf CellTram Vario microinjector. After injection, oocytes were cultured in CZB+IBMX for 0.5-2 hours followed by laser-scanning confocal microscopy analyses (Carl Zeiss).

Confocal microscopy

Immunostaining and Triton extraction procedures for oocytes have been described previously (Kim et al., 2010). For Cos-1 cells, cells were fixed with -20°C methanol for 2 mins, and washed in PBS. Cells were then permeabilized in 0.5% Triton X-100 for 10 min, washed, and incubated with rabbit anti-MATER (1:500) or anti-alpha Tubulin (1:500, Sigma) in PBS with 1% BSA followed by 1hour incubation with appropriate Alexa Fluor conjugated secondary antibodies

(1:500). Cells were mounted on slides with Fast Gold antifade agent (Molecular Probes) and imaged using a Confocal Microscope (Zeiss). For live staining of oocytes, MII oocytes were stained with ER tracker (Molecular Probes) for 25 minutes in MEM-alpha supplemented with 5% FBS, washed, and imaged.

Statistical analysis

All experiments are repeated for at least three times. The intensity of Fluo-4 was normalized to baselines and calculated using ImageJ. Values are given as mean ±STDEV. Values of amplitude and frequency were analysed using student's t-test. Differences of $p<0.05$ were defined as significant.

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CHAPTER IV

SETTING THE STAGE FOR FERTILIZATION:

TRANSCRIPTOME AND MATERNAL FACTORS

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Introduction

Toward the end of oocyte growth, an oocyte matures so that it can be fertilized by a sperm and develop into an early embryo. Oocyte maturation encompasses two main interrelated developmental programs, nuclear maturation and cytoplasmic maturation (1). Nuclear maturation involves the progression from prophase I of meiosis to metaphase II and can be visualized microscopically as germinal vesicle breakdown (GVBD), spindle formation, chromosomal condensation/segregation, and polar body extrusion. Cytoplasmic maturation prepares the oocyte for activation and early development and is characterized structurally by the dramatic redistribution of endoplasmic reticulum (ER) and mitochondria from a relatively diffuse localization in germinal vesicle stage oocytes to a much more polarized distribution pattern in mature eggs. The targeting of mitochondria around the spindle apparatus is thought to provide energy, in the form of ATP, to drive processes such as chromosome segregation while the targeting of the ER to distinct cortical clusters underneath the microvillar cortex is believed to be required for the generation of repetitive Ca^{2+} transients that are necessary for activation of development (2,3). At the molecular level, cytoplasmic maturation involves the accumulation and processing of mRNA and proteins that are also required for successful activation and early development (4). Microtubules (MTs) play a central role in orchestrating the events of nuclear maturation and are also thought to be important mediators of organelle redistribution during cytoplasmic maturation (5). Once mature, the oocyte is then competent for fertilization. Sperm-egg interaction is a sequential process whereby sperm first passes through the cumulus cell extracellular matrix, binds to and penetrates the zona pellucida, and then binds to and fuses with the oocyte plasma membrane (oolemma) (6). The molecular mechanisms behind each of these interactions is now coming to light, thus greatly increasing our understanding of the fertilization

process in mammals. Due, in part, to the transcriptional arrest that occurs following meiotic maturation and prior to activation of the embryonic genome (EGA), the embryo must rely on stores of maternal transcripts and proteins to provide the material necessary to make the oocyte-to-embryo transition (OET) (4). These stored factors are encoded by maternal effect genes (MEG) which can be defined as genes encoded by the maternal genome that are essential early embryogenesis. In light of advanced genomics and proteomics technologies, and with assistance from non-mammalian organisms, over the last 10 years or so, there has been considerable progress in the identification and functional analysis of the role of MEG in mammalian development (7). This chapter will focus on reviewing several aspects of oocyte and early embryo biology during the OET including cytoplasmic maturation, the mechanisms of sperm-egg binding and fusion, and the role of stored maternal factors in mediating early development. Most of the insights into early development that will be highlighted here arose from studies using mouse models.

A. Cytoplasmic maturation

(a) Cytoplasmic organelles

Cytoplasmic maturation is characterized at the organelle level, in part, by the dramatic coalescence of ER and mitochondria around the spindle apparatus and microvillar cortex thus providing localized pools of calcium and ATP for subsequent activation and early development. [We note here that, as opposed to human oocytes where microvilli are more evenly distributed, mouse oocytes contain a microvillus free region which overlies the spindle apparatus]. Below, we will first discuss what is known about mitochondrial, and then ER redistribution during

maturation. We will not discuss the redistribution of other organelles due, in part, to the fact that very little is known about their dynamics at this developmental time-point.

(i) Mitochondria

Oocytes become uncoupled from cumulus cells following ovulation and must sustain their own metabolism through the generation of ATP by mitochondria via oxidative phosphorylation (8). Mitochondria are one of the most abundant organelles in oocytes and embryos must rely exclusively upon maternally inherited mitochondria until the peri-implantation stage, at which point they begin to make their own mitochondrial stores (9). During maturation, mitochondria undergo a highly conserved, but poorly understood reorganization process; with initiation of mitochondrial redistribution coinciding with, and requiring, the resumption of meiosis. In the immature oocyte, mitochondria are more-or-less evenly distributed throughout the cytoplasm and, as the oocyte begins to mature, they coalesce around the germinal vesicle nucleus at GVBD and then become polarized around the spindle during MII stage (2,10). This redistribution event seems to be critical for the production of functional mitochondria and also for the oocyte to reach its full developmental potential. This prediction is based on findings from a range of species which show that a successful mitochondrial redistribution in mature oocytes correlates with increased ATP activity and/or with a higher developmental potential (11-15). Not too surprisingly perhaps, an oocyte's developmental potential is apparently not determined by its total amount of ATP, but instead it is determined by where these ATP pools localize within the oocyte. In other words, to meet the increasing local ATP demands, toward the end of oocyte maturation, subsets of mitochondria at the cortex are thought to become hyperpolarized ($\Delta\Psi_m$). Interestingly, localization of these high potential mitochondria at subplasmalemma has been

correlated with fertilization competency (16). Importantly, mitochondria can be visualized in oocytes using light microscopy (14), thus the non-polarized mitochondrial phenotype in poor quality oocytes may, one day, prove to be an invaluable tool for clinical embryologists to select embryos with high developmental potential for implantation. Regarding the mechanisms mediating maturation-induced mitochondrial redistribution, a key driver of this process is thought to be the fraction of microtubules that are not directly associated with the meiotic spindle apparatus (5,15,17). The methods by which mitochondria are trafficked along MTs in somatic cells are well documented and involve MT-motors such as the ATPase, kinesin, and dynein (18,19). However, much work remains to be done before similar conclusions can be drawn in oocytes. In addition to MTs, microfilaments have also been found to play a role in mitochondrial transport in oocytes. Recently, for example, Yu et al, found that mitochondrial redistribution closely coincided with changes in mitochondrial ATP synthase activity during maturation. Surprisingly, while nocodazole-mediated MT depolymerization was found to inhibit the perinuclear accumulation of mitochondria during GVBD, this drug did not affect ATP production. However, cytochalasin B (which depolymerizes actin filaments) both suppressed the formation of mitochondrial clusters and also suppressed ATP production (20). These strong correlative findings suggest that microfilaments are important for the positioning of functional mitochondria in mature eggs and that mitochondrial clustering may be prompted by increasing energy demands at specific subcellular locations during oocyte maturation.

(ii) Endoplasmic reticulum

Mammalian development is initiated when a sperm delivers the oocyte activating factor, PLCζ, into the egg cytoplasm at fertilization. PLCζ then cleaves PIP_2 to IP_3 and IP_3 binds to the IP_3R-I

on the ER which induces the opening of ER Ca^{2+} channels and subsequent release of Ca^{2+} into the cytosol. The resulting $[Ca^{2+}]_i$ oscillations are required for the activation of development, a process which includes; meiotic resumption, cortical granule exocytosis, and the initiation of embryonic development (21). Prior to fertilization, the capacity for IP_3-induced Ca^{2+} release increases throughout oocyte maturation, with oocytes finally acquiring the ability to initiate fertilization-like oscillations late in maturation (22). This upregulation of the Ca^{2+}-releasing machinery likely depends upon a number of factors including changes in Ca^{2+} stores, changes in Ca^{2+} homeostasis, IP_3R-I functionality, and/or the distribution of ER (23). Regarding the last point, the dramatic redistribution of ER during maturation is seen in a range of vertebrate species and the close correlation between ER positioning and functional $[Ca^{2+}]_i$ oscillations is well documented (24). For example, a spatiotemporal correlation was found between the release of Ca^{2+} and the appearance of IP_3R-rich ER clusters at the cortex in mature MII stage oocytes (3,25). These clusters form at the vegetal cortex, which is the initiating site for $[Ca^{2+}]_i$ oscillations. Thus, the appearance of ER clusters at this site is likely the cause of increased IP_3 sensitivity (26). As with mitochondria, microtubules and microfilaments appear to be responsible for ER redistribution during maturation. Additionally, similar to mitochondria, the ER undergoes two major rearrangements during maturation, with the ER first congregating around the nucleus prior to GVBD, followed by the targeting of ER to the microvillar cortex in the mature egg. Based on inhibitor studies, the targeting of ER to the perinuclear region at GVBD appears to require microtubules and the MT motor, dynein, while targeting of ER to the cortex in the mature egg appears to primarily be mediated by microfilaments (27).

(iii) ER-mitochondrial clustering

ER and mitochondrial clustering during oocyte maturation is associated with developmental potential. Electron microscopic and confocal analysis of oocytes finds that mitochondria appear to undergo an ordered clustering during maturation and also come into close proximity with ER during this time (9). In somatic cells, a number of studies have found that close physical interactions between ER and mitochondria facilitate organelle crosstalk via coordinated signaling events. For example, mitochondrial-derived ATP was found to activate ER membrane Ca^{2+} pumps, which, in turn, propagated Ca^{2+} signals to mitochondria, thus altering their metabolic rate. Additionally, mitochondria can act as a Ca^{2+} buffer by regulating both local Ca^{2+} concentrations and the rate of Ca^{2+} signaling events (28). Based on these (and other) observations, it seems likely that a functional interplay exists between ER and mitochondria in oocytes and that this relationship modulates both Ca^{2+} homeostasis and ATP supply (29,30). Additionally, the coordinated crosstalk between these two organelles in oocytes may prove to be key determinate of successful developmental outcomes in early embryos (31).

(b) Molecular inventory

(i) Fate of transcripts

In light of our extended discussion on organelle redistribution during maturation above, here, we will discuss the molecular aspects of cytoplasmic maturation. During their extended growth phase, mammalian oocytes increase in size from ~20 to ~80 μm, and, during this time, RNA is synthesized and stored at relatively high rates (32). Toward the end of oocyte growth, the rate of transcript synthesis slows and mRNA degradation then commences upon oocyte maturation. Degradation of the majority of mRNAs is accomplished during maturation and is almost

complete in 2-cell embryos (33). The fate of mRNA is often correlated with the length of the transcript's poly(A) tail, with some polyadenylated mRNAs being immediately translated into proteins, which may then be stored for later use by the oocyte during or following maturation and fertilization. Interestingly, it appears that a good many mammalian MEG products fall into this category. For example, proteins encoded by most of the MEGs discussed below, are actually first expressed during the early stages of oocyte growth. Additionally, proteomic studies have found that the mass and isoelectric point of a number of maternal proteins are altered at this time, suggesting that some of these stored proteins are activated during meiotic maturation by post-translational modifications such as phosphorylation (34). Transcripts that are not immediately translated are often deadenylated and subsequently either degraded during maturation or are masked from degradation and then stored in ribonucleo protein particles. These dormant transcripts can then be re-adenylated and recruited for translation at later timepoints (35). Regulation of these activities is mediated in part, by the transcript's 5' and 3' untranslated regions, which can associate with different regulatory factors such as CPEB (36). Additionally, a role for small non-coding RNAs in regulating this activity is now coming to light with, for example, siRNAs being found to modulate maternal mRNA degradation (37).

(ii) Role of PADI6/Cytoplasmic lattices in organelle redistribution

While the redistribution of organelles during oocyte maturation has been reported in diverse arrays of species, the mechanisms by which the cytoskeleton mediates this dynamic event remain poorly defined. As noted, most studies in this area treat oocytes with drugs (such as taxol or cytochalasin B) that alter microtubule or microfilament dynamics and then observe the effects of these drugs on organelle distribution (20,27). Few studies have actually directly observed the

proximate relationship between organelles and the cytoskeleton during maturation, nor have many studies investigated the molecular mechanisms involved in tethering organelles to microtubules or microfilaments. Given that the dynamic redistribution of organelles during oocyte maturation is occurring within an exceedingly large volume (relative to somatic cells), it stands to reason that the oocyte and early embryo may have evolved a specific structure to help facilitate organelle positioning and distribution. Recent studies suggest that the oocyte cytoplasmic lattices may represent just such a structure. The cytoplasmic lattices (also called cytoskeletal sheets, plaques, lamellae, and fibrillar arrays) are absent from non-growing oocytes but increase in number dramatically throughout oocyte growth, eventually becoming a dominant feature of the fully grown mouse oocyte (38). Electron microscopic analysis of the oocyte lattices finds that these structures are composed 5-7 ~20 nm filaments lying side by side with the filaments appearing to be held in place by cross-bridges spaced every 23-25 nm (39). Regarding the molecular composition of the lattices, previous studies suggested that the lattices represented a storage site for yolk, ribosomes, or possibly intermediate filaments (40-42). More recently, immuno-EM analysis found that that peptidylarginine deiminase 6 (PADI6), a highly-abundant oocyte and embryo-restricted maternal protein, localizes to the lattices (43). Regarding expression, PADI6 protein is observed in the oocyte cytoplasm as soon as oocytes enter their growth phase and the protein persist in embryos up to the blastocyst stage of development, which is similar to that of the lattices. Analysis of *Padi6*-null oocytes found that lattices are not observed in mutant oocytes at any point in oocyte growth or in early embryos, suggesting that PADI6 is a component of the lattice complex and that, in the absence of PADI6, the lattices do not form (44). In addition to PADI6, two other maternal factors, MATER and FLOPED, have also been found to localize to, and be required for, lattice formation (45,46). MATER is a

member of the NLRP gene family and contains five tandem hydrophilic repeats at its N-terminus, a NACHT-NTPase domain within its core region, and a leucine-rich repeat (LRR) domain at its C-terminus (47). The functional significance of these domains within MATER has yet to be explored. FLOPED encodes an atypical KH-domain RNA binding domain (48). The expression patterns for both MATER and FLOPED in oocytes and early embryos are similar to PADI6 (43,49,50). Regarding a role for the lattices in early development, a recent study has found that alpha tubulin interacts with PADI6 at the oocyte lattices and that tubulin solubility is greatly increased in PADI6-null oocytes (17). These and other findings raise the possibility that the lattices may play a direct role in microtubule-mediated organelle distribution during oocyte maturation. This prediction was supported by the observation that both mitochondria and ER fail to undergo maturation-induced redistribution in PADI6-null oocytes. Further, the diffuse organelle distribution pattern observed in mature *Padi6*-null oocytes was not affected by taxol-mediated microtubule hyperpolymerization, suggesting that loss of *Padi6* results in an uncoupling of organelles from microtubules. Additionally, levels of acetylated tubulin, a marker for stable MT, were strongly suppressed in *Padi6*-null oocytes, suggesting that the lattices either contain, or are associated with stable microtubules (17). Interestingly, a recent publication examined whether mitochondrial distribution or function was altered in mutant *Mater* oocytes during maturation. Indeed, mitochondria were found to be distributed in a non-polarized fashion and mitochondrial activity was also disrupted in mutant metaphase II-arrested oocytes (51). Taken together, these findings raise the possibility that the lattices represent a novel form of stable microtubules that have evolved within the oocyte cytoplasm to facilitate organelle positioning and redistribution in oocytes and possibly early embryos.

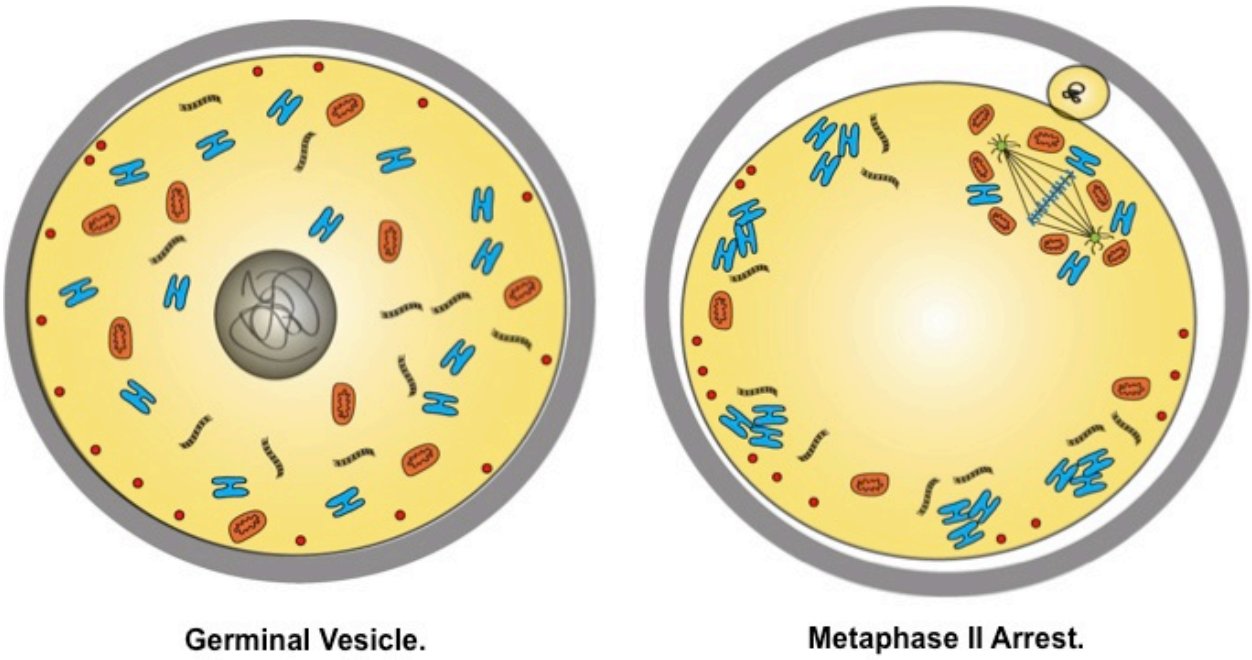

Figure 4.1. Organelle redistribution during oocyte maturation.

In the fully grown prophase I-arrested germinal vesicle stage oocyte, the endoplasmic reticulum (blue) and mitochondria (orange) are diffusely distributed throughout the cytoplasm while cortical granules (red) localize to the subcortex. The germinal vesicle nucleus is shown in grey and oocyte lattices are shown as black fibers. In the mature metaphase II-arrested oocyte, the endoplasmic reticulum is targeted to clusters opposite to that of the spindle apparatus while mitochondria are found to be clustered around the spindle with some hyperpolarized mitochondria being found at the subcortex. Microtubule organization centers (MTOCs) are shown in green.

B. Fertilization: The role of egg proteins in sperm-egg binding and fusion

Mammalian fertilization occurs in a stepwise fashion and initiates when sperm come into contact with, and then penetrate, the oocyte's vestments; the cumulus mass and the zona pellucida (ZP). Sperm then enter the perivitelline space and the sperm's plasma membrane then binds to, and fuses with, the microvillar region of the oocyte's plasma membrane. Following fusion, in addition to releasing PLCζ, sperm also release their highly condensed paternal genome into the oocyte cytoplasm whereby the sperm chromatin deconsenses and the male and female pronuclei form. These pronuclei then are apposed and the two parental genome's unite, thus completing the fertilization process.

(a) Interaction of sperm with the egg vestment

The cumulus oocyte complex (COC) consists of the ZP-encased oocyte and the surrounding layers of cumulus oophorus cells. Cumulus cells secrete a robust extracelluar matrix and it is this matrix that sperm must first traverse to reach the ZP. While cumulus-free oocytes can be fertilized *in vitro*, a number of genetic studies have found that deletion of genes that are involved in stabilizing the cumulus cell matrix, such as *Ambp*, *Tnfip6*, and *Ptx3*, can affect COC integrity and also suppress female fertility (52). Therefore, the passage of sperm through the cumulus matrix appears to facilitate the fertilization process *in vivo*. While the precise role that cumulus cells play in fertilization remains unclear, several reports suggest that the cumulus matrix may promote the acrosome reaction (52). Following penetration of the cumulus cell matrix, sperm then interact with, and pass through, the ZP. In mice, the ZP is comprised of three glycosylated proteins, ZP1, ZP2, and ZP3 while human oocytes contain a fourth zona protein, ZP4. Numerous older reports indicated that O-glycan carbohydrate moieties on ZP3 appear to function as the

primary sperm receptor and also to induce the acrosome reaction (53). These models also suggested that, following the acrosome reaction, the exposed inner acrosomal membrane of sperm then binds to ZP2 in a secondary reaction that allows for the penetration of sperm through the ZP (54). This long-standing model may now have to be significantly revised as more recent genetic ablation studies in mice suggest that ZP3 is not the primary sperm receptor, but instead the receptor appears likely to be ZP2 (55). This finding could potentially simplify the mechanism of sperm penetration through the zona, with the new model suggesting that sperm-zona binding and penetration is mediated by interactions between sperm and the N-terminus of ZP2 throughout the zona matrix. Additionally, the model posits that, following fertilization, cortical granules exocytose their contents into the pervitelline space and these enzymes then cleave ZP2, thus destroying the zona's sperm receptor, thereby preventing polyspermy. Recently the molecular identity of the one of proteolytic factors responsible for ZP2 cleavage has been identified as Ovastacin (*Astl*), an oocyte-specific metalloendoprotease (56).

(b) Sperm-oolemma interaction

(i) SAS1B

Having penetrated the zona pellicida, the acrosome reacted sperm enters the perivitelline space and then binds to, and fuses with, the oolemma at the microvillar region of the plasma membrane. A number of earlier studies suggested that the initial binding events may be mediated by interactions between integrins on the oolemmal surface and their cognate ligands, the ADAMs (A Disintegrin And Metalloprotease domain), on sperm. However, analysis of knockout oocytes which lacked the various integrin subunits thought to mediate gamete binding, found that

these molecules do not appear to play a role this process (57). Recently, a sperm-specific acrosomal membrane protein, SLLP1, was found to specifically bind to the oocyte plasma membrane and its binding partner on the oolemma was then identified through a panning screen as the oocyte microvillar membrane-restricted metalloproteinase, SAS1B (Sperm Acrosomal SLLP1 Binding) (58,59). *In vitro* studies then found that SLLP1 and SAS1B directly interact and that anti-SAS1B antibodies block fertilization. Lastly, analysis of *Sas1b*-null mice found that these females were subfertile. These new studies provide the first defined pair of molecules (i.e. sperm membrane SLLP1 and oolemmal SAS1B) that appear to specifically mediate sperm-oolemmal binding, but do not appear to play a role in subsequent fusion events.

(ii) Tetraspanins

In addition SASB1, tetraspanins, and glycosyl-phosphatidylinositol-anchored proteins (GPI-AP) on the oolemma have also been found to play important roles in sperm-egg binding and fusion. Tetraspanins are known to function as "molecular facilitators" by bringing together and stabilizing molecular complexes called tetraspanin webs which form between tetraspanins and other transmenbrane proteins and also associate with signaling pathways and cytoskeletal elements (60). The first clues that tetraspanins may play an important role in sperm-oocyte membrane binding and fusion, came from a study in the late 1990's which found that a function blocking antibody to the tetraspanin, CD9, strongly suppressed sperm-oocyte binding and fusion in a dose-dependent manner (61). A number of subsequent studies in *Cd9* null mice found that, despite its nearly ubiquitous expression pattern, the only discernible phenotype in mutant mice, was that female mice were severely subfertile and that, while sperm could bind to *Cd9* null oocytes, fusion was almost completely blocked (62-64). Results from these mutant mouse

studies suggested that CD9 is primarily involved in membrane fusion, however, more recent studies have found that CD9 may also play a role in sperm-oocyte binding, thus supporting the initial anti-CD9 function-blocking antibody study (65). In addition to CD9, genetic and molecular analysis of another tetraspanin, CD81, have found that this protein also appears to play an important role in sperm-egg interaction, however, the defects observed in *Cd81* mutant oocytes are less severe (66). It is possible that these tetraspanins are directly involved in mediating sperm-oocyte interaction by, for example, functioning as a receptor for a sperm ligand. However, complementary sperm ligands for CD9 have yet been identified.

(iii) The potential mechanism by which CD9 regulates sperm-egg interaction

While CD9's putative sperm ligand remains unknown, the best candidate ligand for mediating sperm-oolemmal interaction on the sperm side is the sperm-specific protein IZUMO1. Analysis of *Izumo1* mutant mice finds that males are infertile and that, while *Izumo1*-null sperm can penetrate the ZP, they cannot bind or fuse with the oocyte plasma membrane (57). In an ideal world, CD9 would be found to be the receptor for IZUMO1, however, an interaction between these two proteins has not been demonstrated and the receptor for IZUMO1 remains to be determined. An alternate indirect mechanism by which CD9 could facilitate sperm-oolemma interaction, is by regulating the structural landscape of the oolemma in *cis*. For example, CD9 may interact with other molecules in the oocyte plasma membrane to form a tetraspanin-enriched microdomains that are required for sperm-egg interaction. In support of this prediction, previous studies found that pre-incubation of eggs, but not sperm, with the large extracellular loop of CD9, which tetraspanin-associated proteins normally bind, hampered gamete fusion (67). Another indirect mechanism by which CD9 may regulate sperm-egg interaction is by mediating

the microvilliar architecture. A recent electron microscopy study found that CD9 localizes to oocyte microvilli and that the microvillar morphology of *Cd9*-deficient oocytes is altered in length, density, and thickness (68). Lastly, other studies have found that oocytes appear to release CD9-containing exosomes that can bind to sperm and thus facilitate sperm-egg fusion by allowing for direct interactions between sperm-localized CD9 and oocyte-localized CD9 (69).

(iv) GPI-anchored proteins

Glycosyl-phosphatidylinositol-anchored proteins (GPI-APs) on the oolemma have also been found to be required for sperm-egg binding and fusion. A role for GPI-AP in sperm-egg interaction was identified when investigators found that treatment of mouse oocytes with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves the lipid tether in GPI-anchored proteins, significantly diminished the ability of the egg to bind to, and fuse with sperm. This effect appeared to be limited to oocytes as PI-PLC-treated sperm could fertilize oocytes at a normal rate (70). This study was supported by another report which asked a similar question using a mouse genetic model system. These investigators generated an oocyte-specific knockout mouse lacking the *Pig-a* (Phosphatidylinositol glycan class-A) gene, which encodes the enzyme that catalyzes the first steps of GPI-anchor biosynthesis. Results showed that oocytes which lacked the *Pig-a* gene matured normally, yet possessed a significantly reduced ability to fuse with sperm (71). To date the molecular identity of the GPI-anchored protein mediating these events remains unknown.

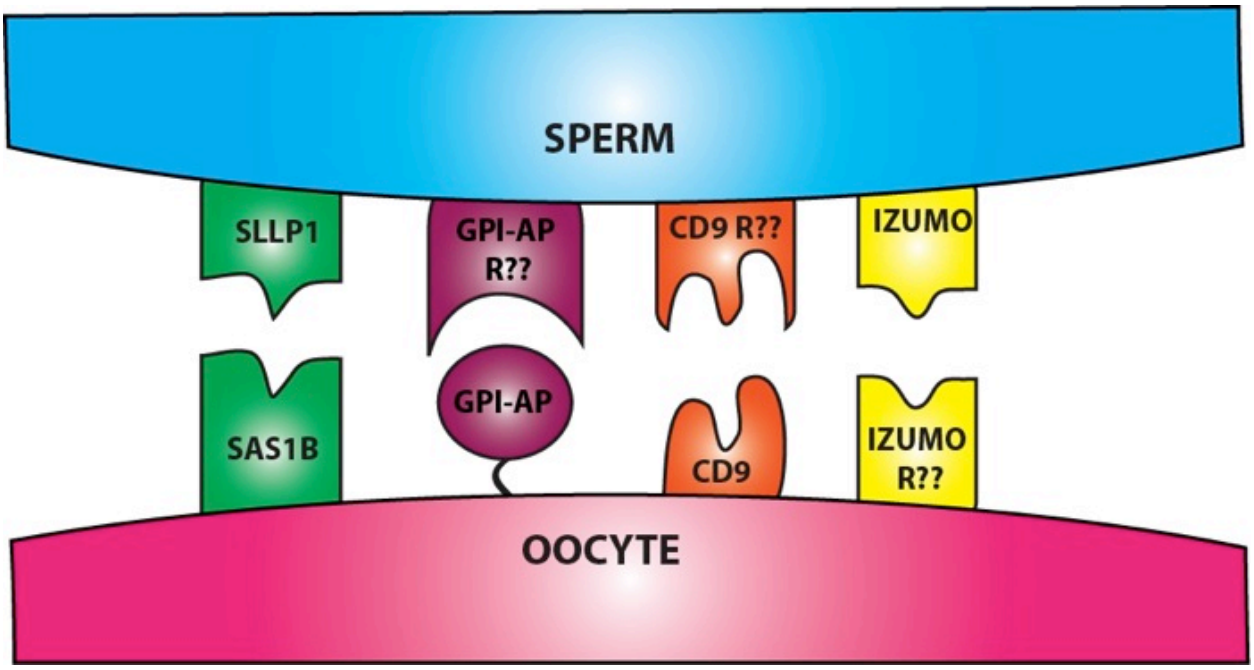

Figure 4.2. Molecular "players" involved in sperm-oocyte binding and fusion.

Sperm SLLP1 has recently been shown to directly interact with oocyte-restricted SAS1B, thus defining the first pair of molecules known to mediate sperm-oolemma binding. On the sperm side, IZUMO has been shown be required for sperm-oocyte binding and fusion, however its cognate receptor on the oolemma is not known. On the oocyte side, GPI-anchored proteins (GPI-AP) and CD9 have been found to be required for sperm-oocyte binding and fusion, however, their putative cognate receptors on sperm have not been identified.

C. Maternal effect genes

In the final section, we will briefly discuss a panel of maternal factors stored within the oocyte that facilitate the OET. These genes fit well within the category of maternal effect genes because, while their gene products are synthesized by the oocyte, these factors are essential for early development. In this summary, we will focus on germ cell-specific maternal effect genes, as it seems likely that factors that have evolved specifically to mediate effects in the egg and early embryo and are likely to be fundamentally important for early development.

(a) Chromatin remodeling

(i) NPM2

Maternal and paternal chromatin undergoes remodeling upon fertilization to ensure proper formation of the diploid genome. Following entry into the oocyte cytoplasm, decondensation of paternal chromatin occurs over a 30 min to 1h time period (72). In *Xenopus*, this process is thought to be largely mediated by histone chaperones such as nucleoplasmin (NPM) and nucleosome assembly protein 1 (NAP1) (73,74). In mammals, NPM2 protein expression initiates in growing oocytes and persist until the blastocyst stage. NPM2 predominantly localizes to the nucleus of GV stage oocytes, is dispersed throughout the cytoplasm following GVBD, and then translocates back to the nucleus upon pronuclear formation; with nucleocyoplasmic shuttling potentially being mediated by phosphorylation (34). *Npm2*-null females are subfertile with ~60% of embryos arresting around the 2-cell stage of development. In mammals, defects in decondensation of the sperm nucleus were not observed in zygotes derived from *Npm2*–null females, thus NPM2 was originally thought not to play a role in sperm nucleus decondensation.

However, a recent report found that removal of the nucleolus-like body from GV stage oocytes slowed sperm decondensation and that microinjection of NPM2 mRNA partially rescued this defect (75). A potential role for NPM2 in sperm decondensation was further supported by the observations that sperm decondensation was retarded in zygotes derived from *Npm2*-null females and that recombinant NPM2 could induce sperm decondensation *in vitro*. In addition to sperm decondensation defects, nuclear and nucleolar abnormalities were detected in null zygotes as were defects in histone deacetylation and heterochromatin formation, thus further supporting a role for NPM2 as a histone chaperone (76).

(ii) ZAR1

*Zar1 (*Zygote arrest 1) is conserved across various vertebrate species including zebrafish, frog, mice, and humans. In mice, ZAR1 protein expression initiates in growing oocytes and persist until the 2-cell stage of development. *Zar1*-deficient females are sterile, with embryonic arrest occurring at either the pronuclear stage (due to the zygote's inability to form a diploid genome) or at the two-cell stage (due to a suppression of embryonic transcription) (77). ZAR1 contains an atypical plant homeobox domain (PHD) that is found in many transcription factors, thus suggesting that this maternal protein may play a role in gene regulation (78). Additionally, ZAR1 was recently found to interact with its homolog ZAR1-like, which colocalizes with mRNA processing factors, suggesting that ZAR1 may also regulate RNA metabolism (79).

(b) DNA methylation in pre-implantation embryos

(i) DNMT1o

Following fertilization, a small subset of imprinted paternal and maternal genes are spared from global DNA demethylation during remodeling of the genome in order to preserve parent-specific gene expression patterns in the resulting embryo (80). An oocyte-specific DNA methyltransferase, DNMT1o, has been identified and is now believed to play a critical role in maintenance of these methyl marks. *Dnmt1o* null females are almost entirely infertile, with most conceptuses dying between E14-21. Offspring of *Dnmt1o* deficient females exhibit apparently normal global methylation, but display a suppression of methylation at ~50% of allele specific imprints (81). Based on the observation that DNMT1o is targeted to the nucleus of 8-cell stage embryos, this methyltransferase is thought to maintain methylation at imprints at that 8-cell stage. Interestingly, Hirasawa et al. generated conditional knockouts for both *Dnmt1o* and *Dnmt1s* genes and found that the resulting embryos showed a complete loss of methylation at imprinted loci, indicating that, in addition to DNMT1o, DNMT1s also appears to play an important role in maintenance of imprints during preimplantation development (82).

(ii) STELLA

Stella (*Dppa3*) is expressed in primordial germ cells, oocytes, cleavage stage embryos, and pluripotent cells. Analysis of *Stella*-null female mice found that while oogenesis, ovulation, and fertilization appeared normal, these animals were markedly subfertile, due to a developmental arrest in preimplantation-stage embryos (83,84). *Stella* encodes a protein with a DNA binding-SAP motif and a splicing factor domain, thus suggesting this factor plays a role in embryonic gene transcription and/or mRNA processing. Functional studies indicated that, in collaboration with RanBP5, Stella also appears to protect several loci (including imprinted genes) from demethylation, thus implicating *Stella* in maintenance of DNA methylation patterns following

fertilization (85). *Stella* has also been implicated in germ line specification; however, defects of this nature were not observed in mutant *Stella* mice (83).

(c) Subcortical Maternal Complex

In addition to PADI6 and MATER's role in lattice formation and maturation-induced organelle redistribution in oocytes, analysis of mutant mice finds these two maternal factors are also essential for progression of development beyond the two-cell stage (44,86). Likewise, FLOPED (which is required for lattice formation) is also essential for early development (50). Interestingly, MATER and FLOPED were previously identified as members of a high molecular weight complex, named the SubCortical Maternal Complex (SCMC). This complex was also found to contain two other maternal genes, FILIA and TLE6, and was also predicted to contain PADI6 (50). The precise relationship between the lattices and the SCMC is currently being investigated as is the role of the lattices and the SCMC in pre and post-fertilization events. Interestingly, in morula stage embryos, members of the SCMC have been found to localize to the non-opposed cytocortex of outer blastomeres, while apparently being excluded from the inner cells. This restricted localization pattern has led to the hypothesis that the SCMC complex may provide a molecular marker of embryonic cell lineages and possibly play a role in cell fate decisions during early development (48,50).

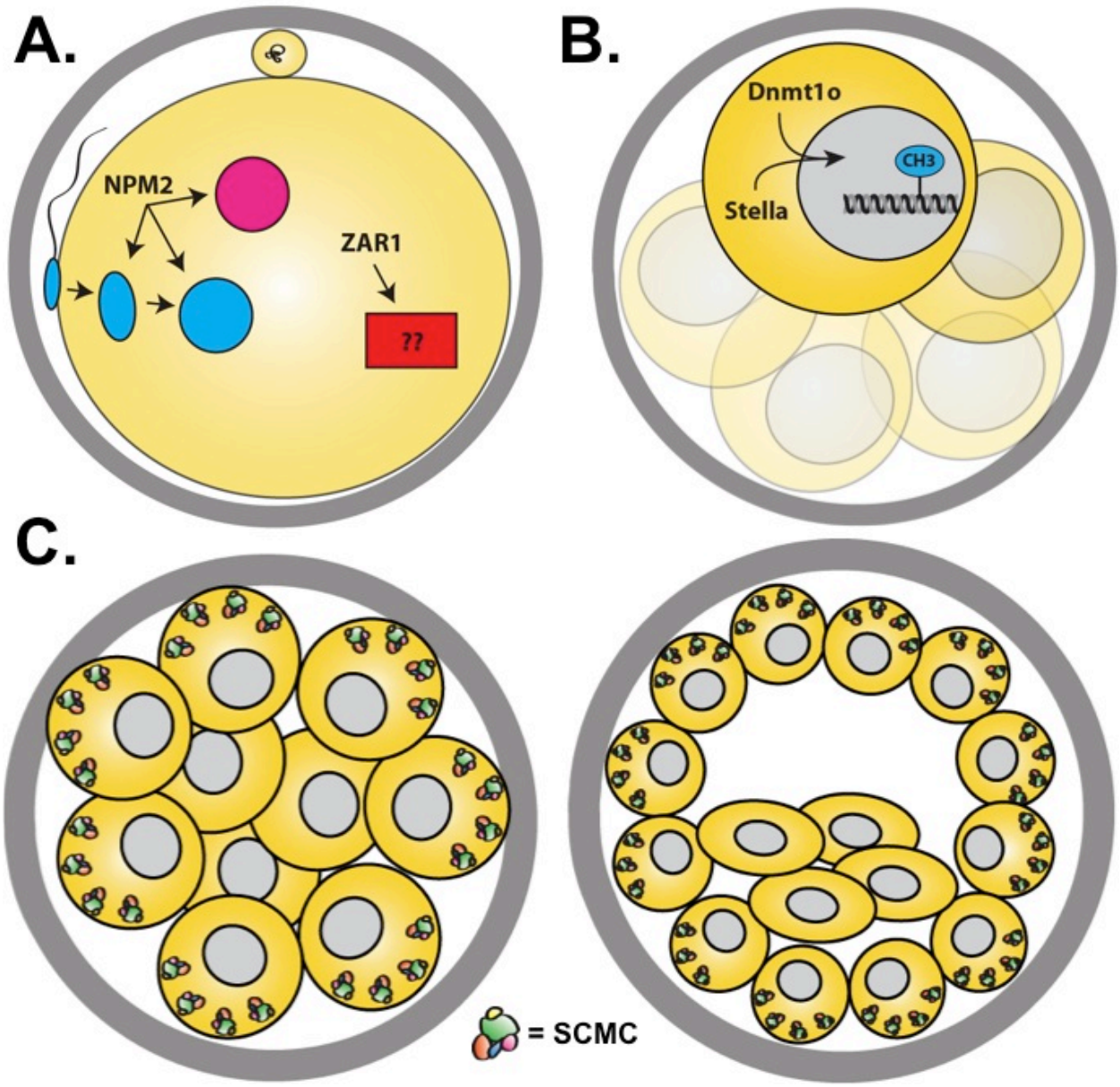

Figure 4.3. Potential role for maternal effect genes in early development.

(A) Nucleoplasmin 2 (NPM2) is targeted to chromatin of decondensing sperm and to male and female pronuclei where, as a histone chaperone, it is believed to play a role in chromatin remodeling. Zar1 is essential for female fertility, however a role for this maternal factor has yet to be defined. (B) Dnmt1o and Stella are both believed to play a role in the maintenance of DNA methylation at imprinted loci. (C) The subcortical maternal complex (SCMC) consists of

MATER, FLOPED, TLE6, and FILIA, with PADI6 being indicated as a putative component. This maternal complex is thought to localize to the non-apposed cytocortex of outer blastomeres and excluded from inner cells, thus potentially playing a role in cell fate determination in the early embryo.

Conclusions and Future Directions

This chapter has focused on reviewing recent advances in oocyte maturation and fertilization and has also discussed the role that stored maternal factors play in mediating the oocyte-to embryo transition. Over the last decade, the use of mouse genetic models and "omic" technologies such as transcriptomics and proteomics have greatly increased our understanding of how the oocyte sets the stage for early development. Perhaps the one of the biggest black boxes that remains to be elucidated in this area is how these events are tied together and regulated. In particular, while the signaling pathways driving meiotic resumption and completion are fairly well fleshed out, the signaling mechanisms that mediate cytoplasmic maturation (structural and molecular) are almost completely unknown. A better understanding of these pathways will help us understand how the egg prepares for fertilization and activation of development. Likewise, while the mechanisms of sperm-oolemmal binding and fusion and oocyte activation are now being elucidated, it seems likely that multitude of additional signaling pathways are activated following gamete fusion, thus further investigations into these events would likely be productive. For example, while CD9 is clearly a critical "player" in sperm-oolemmal binding and fusion, the observation that the tetraspanin web is often associated with actin filaments suggests that CD9 may have additional, yet to be defined, roles in signaling to the oocyte cytoskeleton. Lastly, the recent use of mouse genetic models to investigate the function of a number of oocyte-restricted/abundant gene products has led to the finding that many of these molecules are essential for female fertility via their role in post-fertilization events. Given that many, if not all, of these genes are conserved in humans, a better understanding of these factors and the complexes and structures that they form will undoubtedly aid in our understanding of the oocyte to embryo transition and also potentially shed light on the mechanisms underlying human fertility and infertility.

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